Biological markers in multiple sclerosis related to disease activity and progression

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related to disease activity and progression

M.J. Eikelenboom

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Biological markers in multiple sclerosis related to disease activity and progression

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Voor mijn ouders

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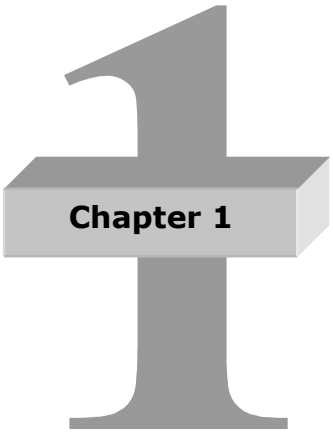


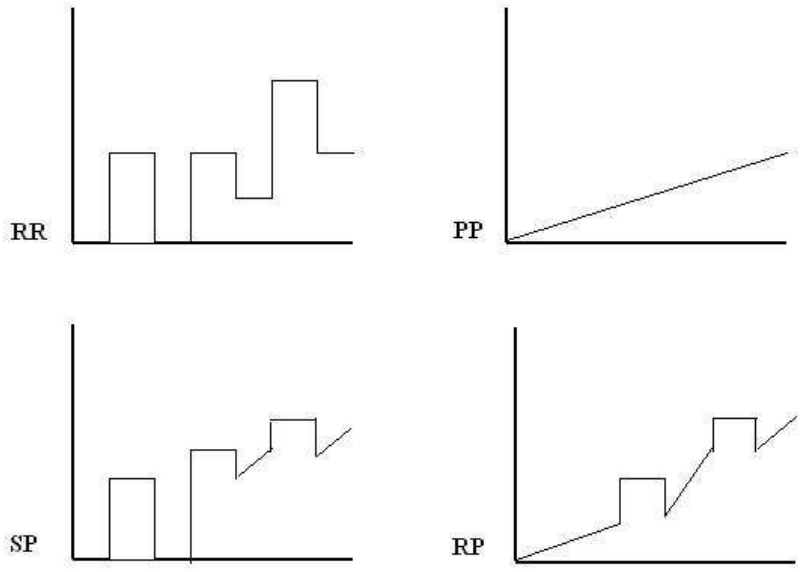
General Introduction

General Introduction

Multiple Sclerosis (MS) is the most common neurological disease in young adults in the developed world with a prevalence of 30 per 100.000 persons (Kurtzke et al., 1991). As in many autoimmune diseases, there is a female predominance (2:1) (Duquette et al., 1993). The clinical course can vary highly both within and between patients. The disease is on base of their clinical course often divided into four subtypes: relapsing remitting (RR), secondary (SP), primary progressive (PP) and relapsing progressive (RP) MS (Lublin and Reingold, 1996; **Figure 1**). Some syndromes have been described monofasic neuritis optica, myelitis transversa, neuromyelitis optica, Marburg´s disease and Balo´s con-centric sclerosis, which may be variants of MS.

Figure 1.

Schematic views of change in level of disability during follow-up for RR, SP, PP and RP patients



The majority of MS patients (85%) initially experience a relapsing remitting disease course and most of them will subsequently develop in a secondary progressive course (Noseworthy et al., 2000). The remaining fifteen percent of the patients have a primary progressive course from onset without relapses. The most common symptoms are: optic neuritis, Lhermitte´s sign, sensory symptoms, limb weakness, brain stem symptoms, Uhthoff symptom and fatigue.
The relapses (clinical exacerbations) or the gradually worsening in the progressive disease eventually lead to a similar fate, fifty percent of the patients require a cane to walk 15 years after onset of disease (Weinshenker et al., 1989). Disability of patients with MS is usually rated using the expanded disability status scale (EDSS) (Kurtzke, 1983). This scale ranges from 0 (no objective neurological impairment) to 10 (death due to MS). However, this scale is highly dependent on the walking distance of patients and therefore additional scales such as the multiples sclerosis functional composite (MSFC) measure (Fischer et al., 1999) have been developed.
Until recently, the diagnosis multiple sclerosis was made by using the diagnostic criteria of Poser (1983). The Poser criteria had as their gold standard for the diagnosis of MS two or more attacks affecting two or more necessarily separate sites within the central nervous system but, for clinically definite disease, also allowed clinical symptoms and signs, to be replaced by paraclinical features (e.g. evoked potential results) and laboratory investi-gations (presence of oligoclonal bands or an elevated IgG index in the cerebrospinal fluid (CSF). Revision of the criteria by an International Panel on MS Diagnosis resulted in new guidelines, the Mc Donald criteria (2001), which were recently revised (Polman et al., 2005; **Table 1**). The main difference with the old criteria is the explicit integration of Magnetic Resonance Imaging (MRI) findings.

Environment and genetics

The cause of MS is not known. Epidemiological studies have shown that environmental and genetic factors contribute to the development of MS. The prevalence of MS increases with distance from the equator (Kurtzke et al., 1980) and clusters of MS have been reported. Migration may change the risk for MS, depending on the age at migration. In addition, several infectious agents have been associated with MS. Recent reports have suggested human herpes virus-6, Chlamydia

pneumoniae and Ebstein Bar Virus, as the most important agents (Challoner et al., 1995; Gilden, 1999; Wekerle et al., 2003), but conflicting data have been published.

Table 1.

The Revised Diagnostic Criteria for Multiple Sclerosis

Clinical Presentation	Additional Data Needed for MS Diagnosis
Two or more attacks [a]; objective clinical evidence of two or more lesions	None [b]
Two or more attacks [a]; objective clinical evidence of one lesion	Dissemination in space, demonstrated by: • MRI [c] *or* • Two or more MRI-detected lesions consistent with MS plus positive CSF [d] *or* • Await further clinical attack [a] implicating a different site
One attack [a] ;objective clinical evidence of two or more lesions	Dissemination in space, demonstrated by: • MRI [e] *or* • Second clinical attack [a]
One attack [a]; objective clinical evidence of one lesion (monosymptomatic presentation; clinically isolated syndrome)	Dissemination in space, demonstrated by: • MRI [c] *or* • Two or more MRI-detected lesions consistent with MS plus positive CSF [d] *and* Dissemination in time, demonstrated by: • MRI [e] *or* • Second clinical attack [a]
Insidious neurological progression suggestive of MS	One year of disease progression (retrospectively or prospectively determined) *and* Two of the following: • Positive brain MRI (nine T2 lesions or four or more T2 lesions with positive VEP) [f] • Positive spinal cord MRI (two focal T2 lesions) • Positive CSF [d]

a) An attack is defined as an episode of neurological disturbance for which causative lesions are likely to be inflammatory and demyelinating in nature. The event (subjective report and objective back up) lasts for at least 24 hours
b) No additional tests are required; however, if paraclinical tests are negative caution for diagnosis MS needs to be taken
c) MRI demonstration of space dissemination
d) Positive CSF determined by intrathecal production of oligoclonal bands or an increased IgG index
e) MRI demonstration of time dissemination
f) Abnormal VEP as seen in MS

A genetic predisposition has been established. Most of all, by showing the higher concordance rate in monozygotic than in dizygotic twin (31% versus 5 %) (Sadovnick et al., 1993). Several genes have been associated in MS. So far, the most consisting finding is that carriers of HLA-DR2 haplotype within the major histocompatibility complex (MHC) have an increased risk to develop MS (Haines et al., 2002).

Pathogenesis in MS

From a pathological point of view MS is a chronic inflammatory disease of the central nervous system (CNS), which leads to lesions with demyelination, astrocytosis and axonal loss. Despite extensive research, the aetiology of the disease is still unknown. For years MS has been considered as one disease. Recent studies suggest that different patterns of inflammation, demyelination and oligodendrocyte destruction are heterogeneous in different patient groups, but homogeneous within a given patient (Lucchinetti et al., 2000), thus indicating that the pathogenetic mechanisms may be fundamentally different.

However, common pathological features are found in MS lesions. It is generally accepted that inflammation contributes to tissue destruction, but it is still unclear whether this immune response triggers the damage, or is a consequence of the disease process.

Immune process/Inflammation

The most widely accepted hypothesis on the pathophysiology of MS is that T helper 1 cells (Th1) recognizing parts of the myelin sheath drive the process and activated autoreactive T cells stimulate the chronic inflammatory process. An intact Blood Brain Barrier (BBB) can allow limited passage of T lymphocytes. The way the activated T cells infiltrate the CNS involves several steps beginning with weak adhesion and rolling on the luminal side of the endothelium of the BBB, followed by firm arrest and subsequent diapedesis across the BBB. The main factors involved in this process are adhesion molecules (such as intercellular adhesion molecule (ICAM), very late antigen 4 (VLA4 or α4 integrin) and lymphocyte function associated antigen-1 (LFA-1); Ransohoff 1999), chemokine-chemokinereceptor interaction (CCL5/CCR5 and CXCL10/CXCR3; Huang et al., 2000) and CD4 MHC class II binding (Wingerchuk et al., 2001). Upon entry into the CNS, the cells produce matrix metalloproteinases (MMP´s), causing degradation of the type IV collagen matrix and

further disruption of the BBB and facilitating the passage of immune cells and antibodies secreted by B lymphocytes.
In the CNS, activated T cells can produce cytokines after interaction with antigen presenting cells (APC) by the trimolecular complex, consisting of the T cell receptor, MHC class II molecules and the antigen (for example myelin components). Depending on the T cells co-stimulatory ligands and cytokine milieu; the trimolecular complex triggers a specific response. Up regulation of the immune cascade results in a pro-inflammatory response by Th1 cells releasing cytokines such as tumour necrosis factor (TNF) α and interferon (IFN) γ, which also activates macrophages. Th2 differentiated cells secreting anti-inflammatory cytokines such as interleukin (IL) 4, IL10 and IL13 down regulate the cellular immune response, thereby promoting B cell antibody production.
As illustrated above in MS a complex cascade of mainly T cell mediated immune processes takes place. Moreover increasing evidence suggests that additional mechanisms are involved in the formation of MS lesions.

Axonal damage

Although already in the late 19th and the early 20th century Charcot and others made the observation that axons degenerate in MS, there is renewed interest in the neuro-degenerative component of the disease after recent publications. These studies demonstrated that already early in the disease axons may be injured or lost. The reduction of axons can be found in both acute and chronic lesions (Bitsch et al., 2000; Ferguson et al., 1997; Ozawa et al., 1994). Recent axonal damage can be visualised by staining the amyloid precursor protein (APP). The APP accumulation in axons is most prominent in early disease and correlated with the extent of T cells and macrophages into the lesions (Kuhlmann et al., 2002), supporting a causal relationship between inflammation and axonal injury. However, axonal damage can also be demonstrated in cortical plaques where inflammation of immune cells is less pronounced (Peterson et al., 2001) and in the normal appearing white matter (Evangelou et al., 2000).
Axonal damage seems to be permanent and probably contributes highly to the irreversible disability in MS patients, especially in the progressive forms, where clinical progression correlates with brain atrophy (Losseff et al., 1996).
The exact mechanism of axonal loss is hardly known; probably it is a multifactorial process. Inflammation might damage the axon directly or via a different pathway that

includes demyelination (Selmaj et al., 1991; Bo et al., 1994; Hohlfeld et al., 1997). A number of cellular and humoral mediators of the immune response have been shown to be capable of damaging axons including T cells, macrophages, antibodies, nitric oxide, glutamate and matrix metalloproteases. Myelin and oligodendrocyte destruction probably precede axonal injury in MS. Oligodendrocyte death can be caused by distal oligoden-drocyte process atrophy or primary oligodendrocyte degeneration (e.g. caused by genetic defects or metabolic impairment in oligodendrocytes) (Rodriquez and Scheithauer, 1994; Lucchinetti et al., 2000). Promising candidates for reflection of axonal damage in blood or CSF are neurofilaments and Tau.

Treatment in MS

The treatment in MS patients involves three approaches: (i) treatment of the acute exacerbation, (ii) disease modifying treatment (DMT) and (iii) symptomatic treatment. For acute exacerbations most often corticosteroids are used. Data suggest that pre-dominantly the speed and not the degree of recovery are affected by this treatment.

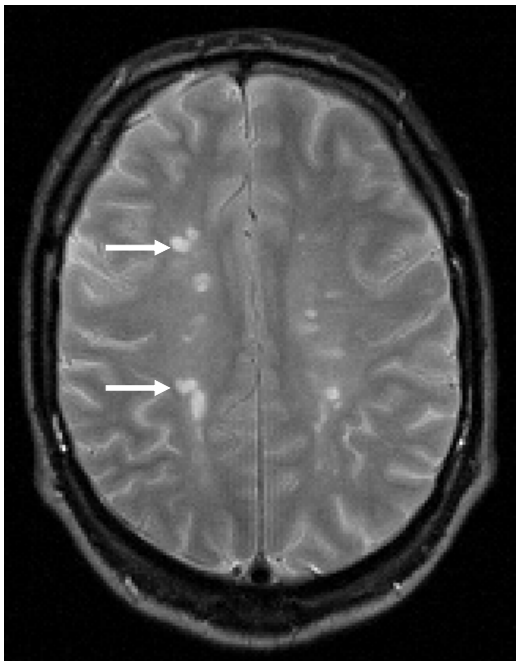
DMT involves several drugs, including interferon β and glatiramer acetate, which both have been proved beneficial in RRMS in large phase III trials and registered in Europe and North America. Blocking pathological cell migration through the blood–brain barrier and into the CNS has long been an attractive therapeutic approach in MS. A promising example is the use of Natalizumab, which binds alpha4-beta1 integrin. Following demonstration of prominent beneficial effects on relapses and MRI it was expedite for approval by the US Food and Drugs Administration (Polman et al., 2006; Rudick et al., 2006). However, a rare but fatal complication associated with the treatment with natalizumab, i.e. progressive multifocal leukoencephalopathy, led to the removal of the drug from the market. Although it is expected that it will be reintroduced, it probably will be with restricted labelling. There is evidence that the use of mitoxantrone in patients with a more aggressive disease course can be beneficial. Furthermore, combination therapy is gaining increasing interest. In clinical care practice, symptomatic treatment is still very important. Spasticity, pain, fatigue, depressions and bladder dysfunction are common symptoms in MS patients that can be effectively treated.

Magnetic Resonance Imaging

The role of MRI in MS has been well established and criteria have been developed to further identify MR features with increased sensitivity and especially specificity for MS (Paty et al., 1988; Fazekas et al., 1988, Barkhof et al., 1997). Since 2001 MRI is included in the diagnostic criteria (McDonald et al., 2001, Polman et al., 2005). MRI also has some prognostic value, particularly in the early phase of the disease and is important in evaluating therapeutic drugs. Furthermore, it has been worldwide accepted that to some extend MRI may reflect the *in vivo* pathological substrate of processes ongoing in the brain. Recent work has shown that T2 (**Figure 2**) lesion load is mainly a marker of total tissue damage and histopathological aspecific (Racke et al., 2001).

Figure 2.

T2 hyperintense lesions on magnetic resonance images, indicated by arrows

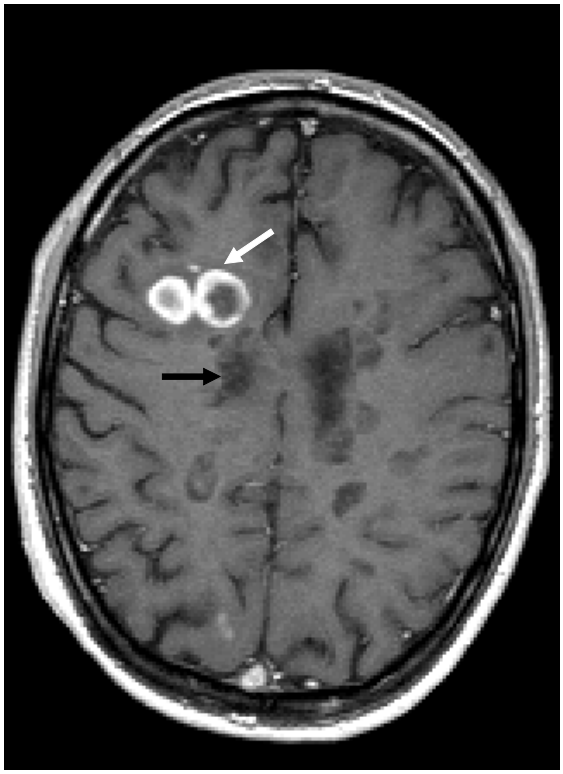


It seems to reflect oedema, axonal loss, gliosis as well as de- and remyelination. Hypointense T1 lesions, the so-called black holes, reflect axonal loss and enlargement of the extracellular space (Bruck, 1997; Walderveen et al., 1998; **Figure 3**).

Gadolinium enhancing lesions have been associated with active inflammation, while it indicates the break down of the blood brain barrier. In addition, atrophy reflected for example by enlargement of the ventricles and reduction of the relative brain volume may indicate the loss of axons and demyelination. Recent observations have shown that using

Figure 3.

T1 hypointense (black arrow) and gadolinium enhancing lesions (white arrow) on magnetic resonance images



more advanced techniques changes can be detected outside the lesions (in the so called ‘normal appearing white matter’, NAWM) already early in the disease (Arnold, 1999). Several post-mortem studies have shown the presence of MS related damage, including axonal and neuronal loss in the cortical and deep grey matter of MS patients (Cifelli et al., 2002; De Stefano et al., 2003). Conventional brain MRI has some limitations in detecting the lesions and damage appearing in white and grey matter. This may be one of the reasons that in clinical practice and research little relationship can be found between MRI abnormalities and clinical disability. Another reason may be that the pathological heterogeneity of MS lesions cannot be identified yet. Techniques to further analyse lesions in grey and white matter as well as the NAWM may be useful to further increase the value of MRI in MS. However, to further

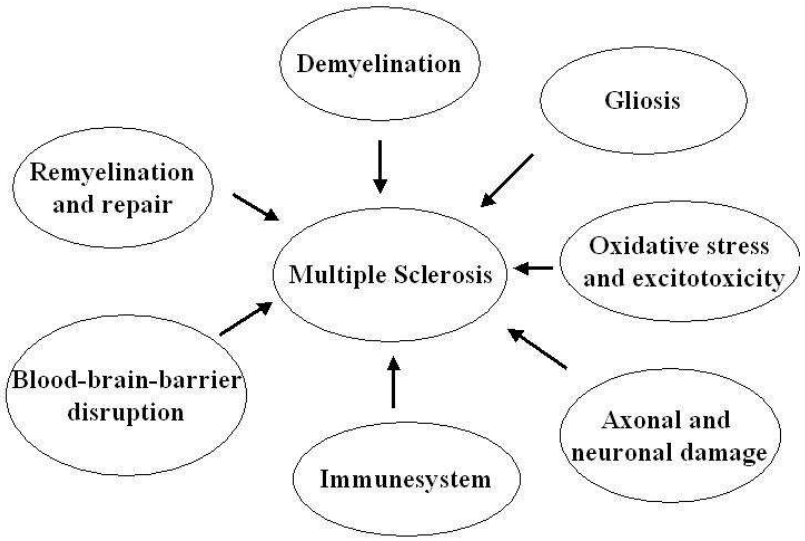
identify the ongoing pathological process in an individual MS patient combination with biomarkers may be warranted.

Biomarkers

As already pointed out, MS is a complex disease in which several pathological processes are involved (**Figure 4**). Furthermore, their relative contribution to the overall disease process is not the same across patients, which may underlie disease heterogeneity with respect to clinical phenotype, prognosis and response to treatment. Successful therapy in MS might involve the combination of different therapeutics targeting various patho-physiological processes. Ideally treatment is individually tailored based on the identification of the dominant process(es). The latter might be achieved by using biomarkers. Also for the development of process-specific therapies biomarkers could play an important role.

Figure 4.

Schematic overview of pathophysiological processes involved in multiple sclerosis



The optimal biomarker is still not found in MS. CSF IgG index and oligoclonal banding are the most consistent markers in MS. In an individual, the same oligoclonal pattern has been consistently seen over time, suggesting a long lived intrathecal

immune response, unique to each individual (Tourtelette et al., 1984; Rolak et al., 1996).

Till recently, research for a biomarker in blood or CSF has been mainly focussed on the immune system. Several candidates have been tested such as cytokines, chemokines, adhesion molecules and antibodies (especially to myelin components). However, none of them have been consistent. This is partly due to the fact that immunological markers are likely to fluctuate for example by other infections in the body, by seasonal variations, by hormones and by relapses. The main emphasis these days in MS biomarker research are on tissue damage. This is based on the expectation that these markers correlate better with the development of long term disability as is the case for MRI markers on axonal damage (Barkhof and Walderveen, 1999). Probably a combination of markers will give us most insight in the pathological processes ongoing in individual MS patients.

Outline of this thesis

The purpose of this thesis was to investigate the role of indicators of ongoing processes in the i) immune system and ii) central nervous system in MS subjects and relate them to clinical and MRI endpoints.

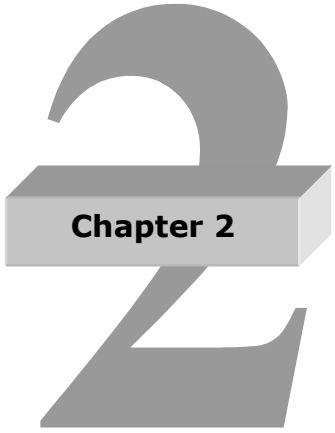
The first part, **Chapter 2,** focuses on the immunological markers in peripheral blood and CSF. **Chapter 2.1** describes the predictive value of the expression of chemokine receptors CCR5 and CXCR3 on T cells in blood for the annualised changes on T2 and T1 lesion load on MRI measurements in MS patients. **Chapter 2.2** describes a cross sectional study in which in a different group of MS subjects, the chemokines CXCL10, the main counter ligand of CXCR3, and CCL2 were investigated in blood and CSF and related to clinical and MRI data. In **chapter 2.3** adhesion molecule expression on T cells of RR, SP and PP MS is correlated to disease progression as reflected by MRI data. **Chapter 2.4** involves a cross sectional study investigating the sex differences between the expressions of cytokines in T cells in MS subgroups. **Chapter 2.5** evaluates the chemokine receptor expression and cytokine production data of peripheral T cells in MS and the opticospinal form of MS.

Chapter 3.1 summarizes the findings of a cross sectional study on glial response markers GFAP, S100B and ferritin and the relationship to clinical findings and a post-mortem study to cross-validate these findings.

Chapter 3.2 and **3.3** focuses on the axonal damage markers in CSF and the association with clinical and MRI data. **Chapter 3.2**, in a cross sectional design, describes the results of the relation between intrathecal production of IgG antibodies against neurofilaments light (NfL) and heavy chain (NfH) and MRI data, such as lesion load and cerebral atrophy. **Chapter 3.3** is an extension, longitudinal design, in which the phosphoforms of NfH in CSF are related to clinical subtypes, clinical scales and progression of disability in a 3 year follow up.

Chapter 4 summarizes briefly the results of this thesis.

In **chapter 5** the additional value of biomarkers is discussed, as well as their role in MS research and clinical practice. Future directions for biomarker research in MS are indicated.



Biomarkers reflecting the immune system

Chapter 2.1

Chemokine receptor expression on T cells is related to new lesion development in multiple sclerosis

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Abstract

The expression of chemokine receptors CCR5 and CXCR3 on CD4 and CD8 positive T cells in blood, measured by flowcytometry, was studied in 124 patients with different clinical subtypes of multiple sclerosis (MS) and 22 healthy controls. In a subgroup of patients (n=69) from whom MRI was available, chemokine receptor expression was correlated to the annualised changes in T1 and T2 lesion load. It was found that CCR5 and CXCR3 on both cell types might have impact on annualised increase in T2 lesion load, but not on T1 lesion load. Our results suggest that chemokines may play a more important role in the development of new lesions in MS than in the long-term outcome of those lesions.

Introduction

Multiple Sclerosis (MS) is an inflammatory disease of the central nervous system (CNS). Current models of the pathogenesis of the disease suggest that systemic activation of myelin-reactive T cells promotes their migration across the Blood-brain-barrier (BBB) where they induce disease by orchestrating inflammatory leukocyte recruitment and activation, with ensuing demyelination and axonal loss. Immunohistological analysis of mononuclear cell infiltration in the CNS has revealed that antigen-specific and non-specific CD4 positive and CD8 positive T cells as well as macrophages constitute the recruited cell population in the CNS, with little or no polymorphonuclear cell in-filtration. The process of leukocyte extravasations into the CNS involves several steps beginning with weak adhesion and rolling on the endothelium of the BBB, followed by firm arrest on the luminal side of the endothelium and subsequent diapedesis across the BBB; this process is governed by both adhesion molecules as well as chemokine-chemokine receptor mediated interaction (Ransohoff 1999).

Chemokines and their receptors are large families of inflammatory molecules responsible for a number of biologic functions including the accumulation of leukocytes at tissue sites. Over the last several years a number of studies have indicated a role for chemokines in the pathogenesis of inflammatory diseases of the CNS. The working hypothesis has been that chemokines induce leukocyte accumulation in the CNS through interaction with specific receptors on the cell surface of T cells, monocytes, and neutrophils. The induction of chemokine expression in the CNS is the result of a complex set of events that include stimuli from infiltrating inflammatory cells as well as endogenous expression. The regulation of tissue-specific chemokine expression is incompletely understood.

In MS, the expression of specific chemokines and chemokine receptors is thought to govern migration of antigen-specific T cells to sites of inflammation, but as yet data on this concept are limited. Th1 polarized T cells express increased amounts of the chemokine receptors CCR5 and CXCR3, thereby suggesting that in particular the two ligand/receptor pairs CCL5/CCR5 and CXCL10/CXCR3 may play a role and that there may be a link between cytokine and chemokine receptor expression that explains why Th1 cells (preferentially) migrate to the CNS in MS (Huang et al., 2000). It has been shown (Balashov et al., 1999) that the expression of both CCR5

and CXCR3 on circulating T lymphocytes is increased in patients with MS compared to controls, and some, but not all, studies reported these increases to be associated with clinical relapses (Misu et al., 2001; Mahad et al., 2002; Sorensen and Sellebjerg, 2001b). It should be recognized, however, that the number of studies addressing the association between chemokine receptor-bearing circulating cells and disease course or disease activity is limited and that the number of patients included is relatively low. The only study correlating chemokine receptor expression with long-term increase in lesion load on magnetic resonance imaging (MRI) was a small pilot study by our group
(Killestein et al., 2001).

The goal of this study was to investigate the expression of CCR5 and CXCR3 on T cells in patients with different clinical subtypes of the disease (RR vs SP vs PP) and compared to healthy controls, to relate the percentage of chemokine receptors to the differentiation of T cells (memory and naïve), and to study the correlation of the expression of these chemokine receptors with changes in lesion load on MRI during a longitudinal follow-up.

Materials and methods

Subjects

MS patients from the outpatient MS clinic at the VU Medical Centre were studied. The patients (n=124) participated in different treatment trials or natural history studies in our department from 1996 till 2000. Blood samples were taken at the start of these studies. Using the classification system by Lublin and Reingold (1996), patients were assigned to the following subtypes: relapsing remitting (RR; n=39), secondary progressive (SP; n=40) and primary progressive (PP; n=45) MS. The SPMS patient group did partially overlap with patients included in the pilot study reported by Killestein et al. Demographic and patient characteristics are shown in **Table 1**. The subjects did not receive any interferon or corticosteroid treatment in the two months before the vena puncture. No clinically relevant infections or relapses were ongoing at the time of blood sampling. For reference we included 22 healthy controls.

MRI analysis

Sixty-nine patients of these patients (21 RR, 25 SP, 23 PP) were selected on the basis of having undergone at least two MRI scans with an interval of several years, without being treated with drugs that have been shown to modify the disease. On average follow up was 3 years. They were scanned on a 1.0 or 1.5 T MRI system (Siemens AG, Erlangen, Germany); T2 (range: repetition time (TR) = 2000-3000 ms, echo time (TE) = 20-50 and 60-100, number of excitations (NEX) = 1) and T1 weighted images (TR = 600 to 700 ms; TE = 15 to 20 ms; NEX = 2) were made. Since the patients participated in different studies, the slice thickness varied slightly (3-5 mm). This was corrected for by calculating the volume of the lesions (area multiplied by the inter slice distance (Kalkers et al., 2001)). Lesion load measurements were performed on a work-station (Sun, Mountain view, California, USA) using semi-automated seed-growing software developed in house, based on local threshold. The total volume of hyper intense lesions on the T2 images and of hypo-intense lesions seen on the T1 weighted images was calculated and a delta lesion load per year was calculated, to correct for differences in the duration of follow up. All raters were trained in measuring lesion load with a coefficient of variation of less than 3.0 %.

Blood samples and laboratory analysis

Venous blood was collected in evacuated blood collection tubes (Vacutainer, Becton Dickinson, Meylan, France) containing sodium heparin. The samples were kept at room temperature and processed within 24 h. Peripheral blood-derived mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll-Isopaque density gradient centrifugation, and cryopreserved immediately. Freshly thawed and properly washed PBMC were incubated on ice with CD4-APC (Caltag, Burlingame, CA, USA) CD8-PerCP (Becton Dickinson, San Jose, CA, USA), CXCR3-PE (Clone 49801.111, R&D Systems, Minneapolis, MN, USA) and CCR5-PE (Clone 2D7, Pharmingen, San Diego, CA, USA) antibodies for 30 min. In 62 of the MS patients (24 RR, 16 SP and 22 PP), we used the following antibodies on CD4-APC and CD8-PerCP cells CD45 RA (Clone 2H4, RD-1-labeled, Coulter, CA, USA) and CD45 RO (Clone Uchl1, FITC-labeled, Dako, Australia). Cells were analysed with Calibur fluorescence-activated cell-sorting scan and Cellquest software (Becton Dickinson, San Jose, CA, USA). T cells were gated according to forward and sidelight scattering properties and CD4 or CD8 expression. Data are given as percentages of CD4 or CD8 positive cells expressing CXCR3, CCR5 or CD45 RA and RO.

Statistical analysis

Technicians blinded for the clinical data of the patients performed laboratory and MRI measurements. Results are presented as mean with ± SD, and MRI data are given in median with interquartile range. One-way ANOVA, using Bonferroni's post hoc test for multiple comparisons, and Kruskal-Wallis test were taken as appropriate to analyse differences between the subgroups. For the correlations tested Spearman and Pearson

r-values are given depending on the distribution of the data. Not normally distributed were the T1 lesion load and the CD4+CXCR3+ T cells. We used a level of significance

of 5%.

Results

Demographics

As shown in **Table 1**, differences could be shown with respect to age, EDSS and disease duration in the subtypes of the study population.

Table 1.

Demographic characteristics of MS subjects (Mean ± SD)

	All (n=124)	RR (n=39)	SP (n=40)	PP (n=45)
M:F	56:68	12:27	26:14	18:27
Age (yrs)	48.9 ± 11.4	39.9 ± 6.9	49.3 ± 8.0	56.6 ± 11.5
EDSS	4.3 ± 2.0	2.2 ± 1.3	4.9 ± 1.1	5.6 ± 1.7
Disease duration (yrs)	10.2 ± 6.5	6.3 ± 5.0	12.4 ± 6.7	11.8 ± 5.9

RR=relapsing remitting; SP=secondary progressive; PP=primary progressive; All=all of the MS patients; n=number of subjects, F=female, M=Male, yrs=years, EDSS=expanded disability severity scale.

Chemokine receptors and T cell subsets

Figure 1 shows a representative result of a FACS plots from one patient. The percentage of CD4 and CD8 positive cells expressing chemokine receptors CCR5 and CXCR3 in the healthy controls, MS patients and the three disease groups are given in **Table 2**. The chemokine receptors CXCR3 and CCR5 were elevated in CD8+ cells in MS patients compared to controls ($p<0.001$ and $p<0.05$, respectively). Examining the different subgroups, significant differences ($p<0.05$) were also found between SP and PP patients with respect to the percentage of CD8 positive cells (higher in SP patients). Between RR and PP patients, a significant difference was shown with respect to the percentage of CCR5 expressing CD8 positive cells (higher in PP patients). When the two progressive subtypes were taken together and compared to

the relapsing-remitting patients, it appeared that progressive patients had a significantly higher percentage of CD8 cells expressing the receptor CCR5 (data not shown). No significant differences could be shown for the other chemokine receptor expressing cells.

Figure 1.

Representative flow cytometric analysis of peripheral blood mononuclear cells for an MS patient. Percentage of CD4 or CD8+ cells (right half) and of CXCR3 or CCR5+ T cells (lower half). Lymphocytes were first gated for FCS and SSC

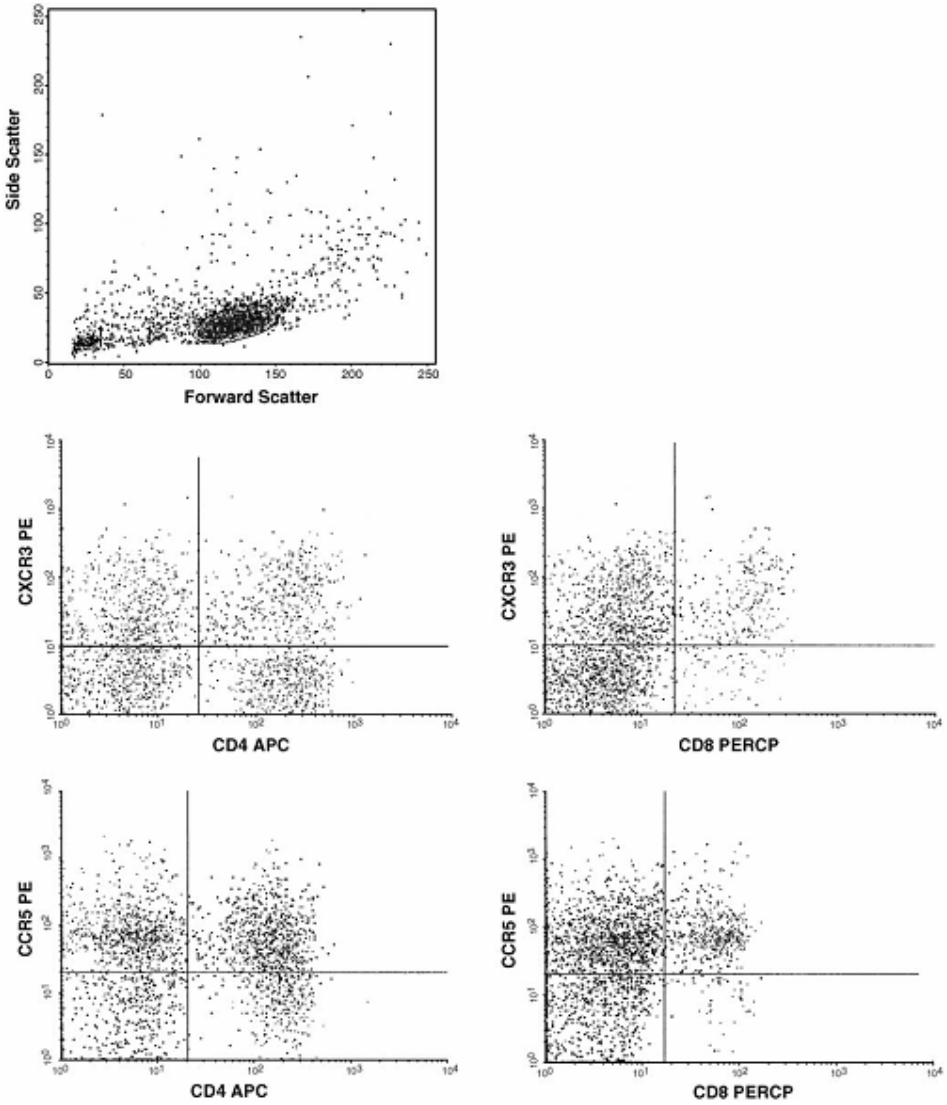


Table 2.

Percentages CD4 and CD8+ T cells and their surface chemokine receptors in 124 MS subjects and 22 healthy controls (mean ± S.D.)

	Con (n=22)	All (n=124)	RR (n=39)	SP (n=40)	PP (n=45)
CD4%	NA	63.6 ± 10.4	62.3 ± 9.7	61.7 ± 10.3	66.2 ± 10.7
CD8%	NA	28.4 ± 8.4	29.6 ± 7.4	30.8 ± 8.5 [a]	25.5 ± 8.4 [a]
% CD4+ CXCR3+	20.5 ± 19.3	23.4 ± 13.8	22.4 ± 12.2	21.6 ± 10.9	25.9 ± 16.8
% CD8+ CXCR3+	30.3 ± 18.0 [b]	46.4 ± 21.9 [b]	48.5 ± 20.9	49.8 ± 22.9	42.1 ± 21.8
% CD4+ CCR5+	11.9 ± 7.8	14.5 ± 9.6	14.9 ± 13.5	14.3 ± 7.3	14.3 ± 7.2
% CD8+ CCR5+	24.8 ± 15.0 [c]	33.7 ± 18.5 [c]	26.9 ± 18.8 [d]	35.7 ± 15.7	37.8 ± 18.8 [d]

Con=healthy controls; RR=relapsing remitting; SP=secondary progressive; PP=primary progressive; All=all of the MS patients; n=number of subjects

a) Difference between SP and PP MS, $p<0.05$
b) Difference between healthy controls and MS patients, $p<0.001$
c) Difference between healthy controls and MS patients, $p<0.05$
d) Difference between RR MS and PP MS, $p<0.05$

In the MS patients, we also determined the percentage of naïve (CD45RA+RO−) and memory T cells (CD45RA−RO+) in the CD4+ and CD8+ subset as shown in **Table 3**. Between the three subgroups, we found a lower percentage of CD4+CD45RA−RO+ in RR MS compared to SP and PP MS patients. In CD8+ cells, we saw lower levels of memory T cells expression in RR MS compared to PP MS. In contrast, naïve CD8+ cells were significantly elevated in RR as compared with SP MS. We correlated the naïve and memory T cells with the chemokine receptors to see which subset of T cells could be accounted for the various expressions of the chemokines. We found significant negative correlations between CCR5+ and CD45RA+RO− in CD4+ T cells ($r=-0.39$, $p<0.01$). The same negative correlations could be found for CD8+

naïve T cells, with CD8+CCR5+ cells (r=−0.27, p<0.05) as well as between CD4+ naïve cells and CD4+CXCR3+ T cells (r=−0.44, p<0.001). In contrast, we saw no significant correlation between memory T cells and the chemokine receptors.

Table 3.

Percentages of naive and memory CD4 and CD8+ T cells in MS subjects (mean ± S.D.)

	All (n=62)	RR (n=24)	SP (n=16)	PP (n=22)
%CD4CD45RA+RO-	45.4±15.0	51.3±12.7	40.7±16.0	42.4±15.1
%CD8CD45RA+RO-	70.5±15.2	77.1±11.4 [a]	63.9±16,6 [a]	68.1±10.2
%CD4CD45RA-RO+	44.9±15.6	37.6±15.5 [b]	49.4±15.6 [b]	49.6±13.1 [b]
%CD8CD45RA-RO+	18.1±11.6	12.7±8.6 [c]	21.1±14.5	21.9±10.2 [c]

RR=relapsing remitting; SP=secondary progressive; PP=primary progressive; All=all of the MS patients; n=number of subjects.

a) Difference between RR and SP MS, p<0.05
b) Difference between RR and SP and SP and PP MS, both p<0.05
c) Difference between RR MS and PP MS, p<0.05

Chemokine receptors and demographic correlations

Significant but weak positive correlations were found for CCR5+CD8+ cells with respect to age (r=0.28, p<0.01), EDSS (r=0.24, p<0.05) and disease duration (r=0.19, p<0.05).

Chemokine receptors and MRI correlation

Table 4 shows the MRI parameters for the three subtypes. **Table 5** and **Figure 2 (A–D)** shows the correlations between the chemokine receptor expressing cells and the annualised change in T2 lesion load. CXCR3+CD8+ cells showed a positive though weak correl-ation with the annualised change in T2 lesion load in the whole group (r=0.35, p<0.01). With respect to the different subtypes, we found a similar, but stronger relationship with the annualised change in T2 lesion load in the SP MS subjects (r=0.68, p<0.001); in this population, we also found a significant

correlation for the CD4+ cells expressing CXCR3 (r=0.56, p<0.01). In RR MS subjects, a significant correlation between CCR5+ expressing CD8+ cells and annualised change in T2 lesion load (r=0.60, p<0.01) was found. There were no significant correlations with the T1 lesion load in the whole group or in the different subtypes for the chemokine receptors (data not shown). Furthermore, the percentage of naïve and memory T cells of CD4+ and CD8+ cells did not correlate with the delta T2 or T1 lesion load per year.

Table 4.

MRI characteristics in subtypes of MS expressed as median (interquartile range)

	All MS subjects (n=69 T2; n=63 T1)	RR (n=21 T2;n=17 T1)	SP (n=25 T2;n=23 T1)	PP (n=23 T1 and T2)
ΔT2LL/ year (cc)	0.22 (-0.13 - 1.5)	0.012 (-0.18 - 1.2)	0.59 (-0.23 - 3.3)	0.20 (0.0.8 - 0.56)
ΔT1LL/ year (cc)	0.06 (0.0 - 0.39)	0.03 (-0.01 - 0.14)	0.40 (0.02 - 0.86)	0.03 (0 - 0.2)

RR=relapsing remitting, SP=secondary progressive, PP=primary progressive, LL=lesion load; n=number of subjects; Δ=delta.

Table 5.

Correlation coefficients between delta T2 lesion load/year and the levels of chemokine receptors on CD4+ and CD8+ cells in 69 MS patients

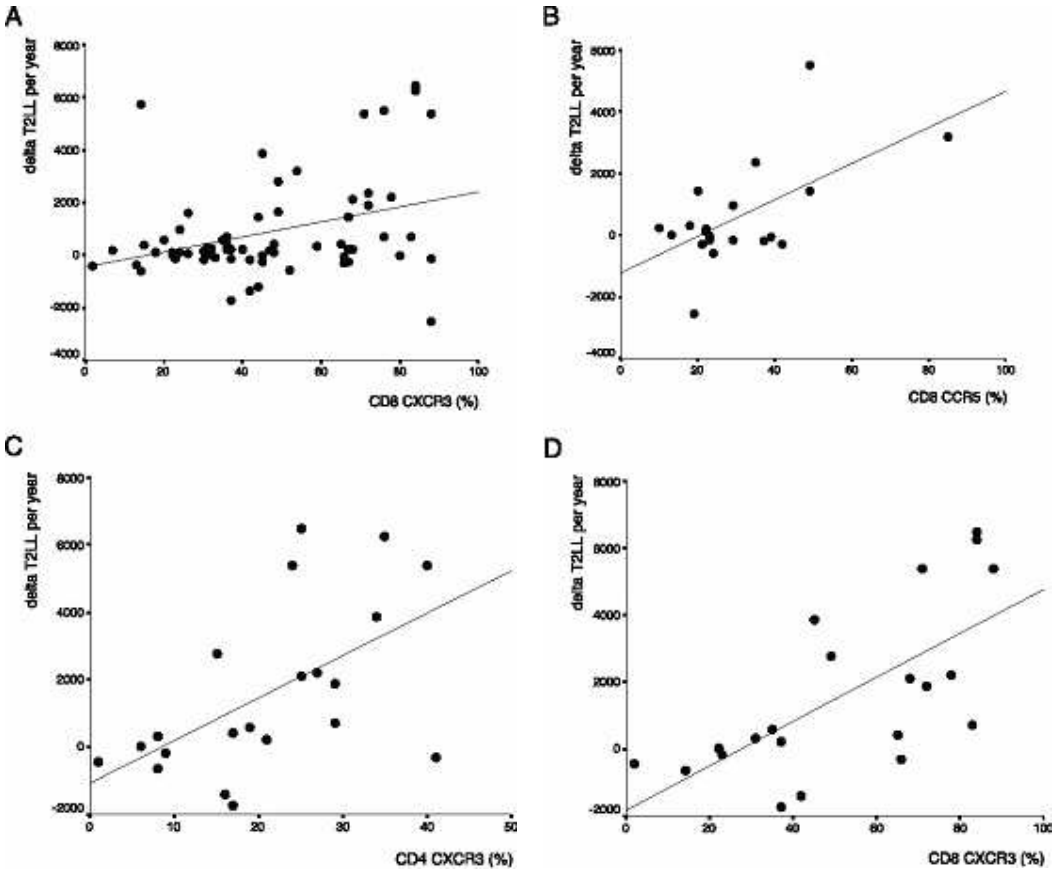
		CCR5+		CXCR3+	
		CD4+	CD8+	CD4+	CD8+
All (n=69)	Δ T2 LL/year	r=0.04	r=0.19	r=0.23	r=0.35 [a]
RR (n=21)	Δ T2 LL/year	r=0.02	r=0.60 [a]	r=0.14	r=0.04
SP (n=25)	Δ T2 LL/year	r=0.22	r=0.20	r=0.56 [a]	r=0.68 [b]
PP (n=23)	Δ T2 LL/year	r=-0.24	r=-0.15	r=-0.01	r=-0.16

RR=relapsing remitting, SP=secondary progressive, PP=primary progressive, All=all of the MS patients, LL=lesion load; n=number of subjects; Δ=delta.

a) Level of significance $p<0.01$
b) Level of significance $p<0.001$

Figure 2. (A-D)

Correlation of delta T2 lesion load/year and the expression of chemokine receptor CXCR3+ on CD8+ cells in MS patients. (B) Correlation of delta T2 lesion load/year and the expression of chemokine receptor CCR5+ on CD8+ cells in the RR subgroup of MS patients. (C). Correlation of delta T2 lesion load/year and the expression of chemokine receptor CXCR3+ on CD4+ cells in the SP subgroup of MS patients. (D) Correlation of delta T2 lesion load/year and the expression of chemokine receptor CXCR3+ on CD8+ cells in the SP subgroup of MS patients



Discussion

Two important messages can be derived from this study. In the first place, we show that CCR5 and CXCR3 expression on CD8 positive lymphocytes is increased in MS patients as compared to controls. More specifically, comparing the different disease groups, we also show that CCR5 on CD8 positive cells is elevated in progressive disease as compared to RR disease. This observation is not completely in line with findings by Sorensen et al. (2001), who found that CCR5 expression was highest in patients with relapsing disease. This difference, however, probably can be explained by the actual disease status of the patients with highest CCR5 expression in their study: these patients were examined during an acute relapse. When comparing our data to the SP MS and RR, "not acute relapse" patients in the study by Sorensen et al. differences were minimal. Remarkably, the highest percentage of CCR5 positive cells in their study was also reported for the CD8 population in SP MS patients. Recently, Jalonen et al. (2002) found a significant increase of intracellular CCR5 RNA expression in PPMS compared to other MS subtypes and controls, in line with our results. Although they did not show a difference with respect to surface expression, they only analysed CD4 positive cells, whereas in our study we analysed both CD4 and CD8 positive cells. Balashov et al. (1999) tested chemokine receptors on CD3+ T cells and found elevated levels of CCR5+ cells in progressive patients compared to healthy control persons. In addition, CD3+CXCR3+ cells, both in RR and in progressive (SP and PP) subtypes, were significantly elevated compared to controls. In this study, a significant difference was described between the RR and progressive groups. Our expression levels of CCR5 are slightly higher and CXCR3+ slightly lower as compared to levels in previous studies (Sorensen et al., 1999), which might be due to the fact that we used cryopreserved cells or that different antibodies were used in the various studies as been pointed out for CCR5 before (Lee et al., 1999). Since we compared chemokine receptor expression data of different subgroups, which have been obtained by using the same standardized methods, we believe that this concern has no impact on the interpretation of the results of our analysis.
Altered immunoregulation is thought to be associated with the disease. Expression of markers for naïve (CD45RA+) and memory (CD45RO+) T cells have also been reported to correlate with the clinical activity of multiple sclerosis (Calopa et al.,

1995; Zaffaroni et al. 1999). Based on our data, we could not explain the differences of chemokine receptor expression in the subgroups or their relation with the T2 lesion load based only on different balance in naïve and memory T cells. Although we found negative correlations between chemokine receptors and naïve T cell, no relation to MRI parameters or subtypes were shown for naïve T cells. The second important observation originating from this study is that correlations were found between the annualised change in T2 lesion load and CXCR3 expression on CD8 positive cells in the total group, CXCR3 expression on both CD4 and CD8 positive cells in SP patients, as well as CCR5 expression on CD8 positive cells in RR patients. These correlations, though statistically significant, are only moderately strong, which is not surprising given the magnitude and complexity of interactions of molecules involved in lesion development, with the best correlation coefficient being 0.68, explaining about 45% of variance of subsequent lesion development. Another factor possibly reducing the strength of the correlations is the fact that MR scans used were collected from patients included in various ongoing studies, resulting in a slight variation in MR techniques and field strength (Kalkers et al., 2001). Although this procedure might have weakened some correlations, in our view, it does not invalidate the main findings of this study.

The positive correlation between annualised T2 lesion load change on MRI and CXCR3 on CD8 positive cells was shown earlier in the pilot study on 14 SP patients by our group (Killestein et al., 2001). Even though we are not aware of studies by other groups which may indicate that CXCR3 expression has long-term prognostic implicat-ions in MS, there are some small studies that have examined the association of chemokine receptor-bearing circulating cells and disease activity or disease course. In one of these studies, it was found that the chemokine CXCL10, which binds the receptor CXCR3, is present in elevated concentrations in the cerebrospinal fluid (CSF) of MS patients in acute relapse (Sorensen et al., 2001). In another study, the number of CCR5 positive circulating CD4 positive cells was studied during relapse and during remission only 3 weeks later: the increase during relapse was followed by a clear decrease, suggesting an association of CCR5 positive T cells with disease activity (Misu et al., 2001).

Maybe our findings should be interpreted as the *in vivo* correlate of post-mortem immunohistochemical findings of CCR5 and especially CXCR3 expression on a large number of perivascular lymphocytes and astrocytes associated with active MS lesions

(Sorensen et al. 1999; Balashov et al. 1999; Simpson et al., 2000). Additional evidence that at least part of this chemokine axis may be important in determining MS disease activity comes from the observations that patients treated with interferon beta have lower serum concentrations of CCL5 and that in families affected by MS, an inactive CCR5 allele was associated with an approximately 3 year delay in disease onset (Barcellos et al., 2000, Iarlori et al. 2000). Additional evidence was seen in individuals heterozygous for the Δ32 mutation, who experienced prolonged disease-free intervals, compared with individuals with a fully functional CCR5 (Sellebjerg et al., 2000).

Both CCR5 and CXCR3 are preferentially expressed on Th1 cells and have been associated with higher secretion of proinflammatory cytokines interferon gamma and tumor necrosis factor alpha that both have been associated with increased disease activity (Sallusto et al., 1998, Balashov et al., 1999; Strunk et al., 2000). As such, our data support the more or less traditional point of view that Th1 CD4 positive T cells are pivotal in initiating disease activity in MS. On the other hand, our best correlations with long-term lesion development have been found for chemokine receptor expression on CD8 positive cells. Recent evidence suggests that especially CD8 positive lymphocytes might be crucial to development of disease progression: CD8 positive T cells have been shown to con-tribute to tissue injury in MS because they infiltrate the CNS parenchyma, and CD8 positive T cell infiltration correlates with axonal injury as assessed by accumulation of amyloid precursor protein (Gay et al., 1997; Bitsch et al., 2000). Biddison et al. have investigated the potential role of CD8 positive cytotoxic lymphocytes in the generation of inflammatory responses in the brain; these authors demonstrated that this subset of cells might be an important source of proinflammatory chemokines that could promote and mediate the inflammatory response in MS (Biddison et al., 1997).

In conclusion, our data expand on existing data that, in particular, the two ligand/ receptor pairs CCL5/CCR5 and CXCL10/CXCR3 may play a role during active disease by showing that expression of CCR5 and CXCR3 on T lymphocytes predicts future disease activity as documented with MRI. Our finding of significant correlations of chemokine receptor expression with change in T2 lesion load (representing new lesions) and not with T1 lesion load (representing more destructive lesions only) may suggest that the chemokine axis is more relevant with respect to initiation than to long-term outcome of lesions, which is in line with current thoughts

on the potential mechanism involved (trafficking of inflammatory T cells into the CNS).

In our opinion, this correlation of chemokine receptor expression with long-term development of new lesions supports ongoing efforts to direct therapeutic interventions towards these molecules (Ransohoff and Bacon, 2000). Our data, with correlations between individual chemokine receptors and lesion development only being moderate, however, may suggest that treatment interventions should address multiple ligand/ receptor pairs.

Chapter 2.2

Chemokines are not related to disease progression as reflected by clinical and MRI measurement in multiple sclerosis

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Submitted for publication

Abstract

The chemokines CXCL10 and CCL2 have been shown to be involved in Multiple Sclerosis (MS). CXCL10 and CCL2 in blood and CSF was analysed in 51 MS patients and related to clinical and MRI measurements of disease state. Our study suggests that CXCL10 and CCL2 should not be seen as markers of disease state in clinical stable MS patients, nor can it discriminate between the subtypes RR, SP and PP.

Introduction

Chemokines have been demonstrated to be involved in Multiple sclerosis (MS), an inflammatory disease of the central nervous system (CNS) (Sorensen et al., 1999 and 2001; Mahad et al., 2000; Franciotta et al., 2001, Scarpini et al., 2002). They can be divided in two main families, based on their arrangement of cysteine residues: α-chemokines, i.e, CXCL10 (formerly know as inducible protein 10, IP-10) and β-chemokines i.e. CCL2 (know as Monocyte Chemotactic Protein-1, MCP-1). Chemokines have been shown to govern migration of antigen-specific T cells to sites of inflammation, but 'in vivo' data on this concept are limited.

CXCL10 is one out of three agonists of CXCR3 and has been associated with Th1 responses, CCL2 is the agonist of CCR2 and capable of inducing the differentiation of both Th1 and Th2 cells (Zlotnik and Yoshie, 2000). CXCL10 and CCL2 mRNA are overexpressed in astrocytes in experimental autoimmune encephalomyelitis (EAE) (Ransohoff et al., 1993). CCL2 is strongly positive in chronic and active MS lesions (McManus et al., 1998). In cerebrospinal fluid (CSF) CXCL10 has been found to be elevated and CCL2 to be decreased in acute MS as compared to non-inflammatory controls. A possible mechanism for the low CCL2 levels in the cerebrospinal fluid of patients with MS is that CCL2 is consumed by circulating mononuclear cells that bear CCR2, the major receptor for CCL2 (Mahad et al., 2006). Furthermore, CSF CXCL10 is positively and CCL2 is negatively correlated with time since last relapse (Mahad et al., 2002). We have previously shown that chemokine receptors (CXCR3 and CCR5) on peripheral T cells might be involved in lesion development in MS as evidenced on magnetic resonance imaging (MRI) (Eikelenboom et al., 2002). So far, only limited data are available on chemokine levels in CSF of MS patients or their relation to lesion load on MRI.

The aim of the present study was to quantify the concentration of CCL2 and CXCL10 in paired CSF and serum samples of MS patients, to compare the different subtypes of MS and to investigate the relation of chemokine levels to clinical and MRI measurements of disease state and disease activity.

Materials and methods

Subjects

Fifty-one (20 relapsing remitting (RR), 21 secondary progressive (SP) and 10 primary progressive (PP)) MS patients with a mean age of 44.5 years and a disease duration of 13.9 years were included in this study (Eikelenboom et al., 2003). They were recruited in response to an appeal in the periodical of the Dutch MS Society and their diagnosis was confirmed using commonly applied criteria (Poser et al., 1983). All the patients underwent a vena and lumbar puncture (LP), extensive neurological examination and a MRI scan within one week. Disability was measured using the Expanded Disability Status Scale (EDSS); the median EDSS of the population was 3.5 (interquartile range (IQR) 2.0-6.5). The median time since their last relapse was in the RR and SP group 36.5 months (IQR 6.3-81.3).

Blood and CSF analysis

Total immunoglobulin G was measured in CSF and serum samples by a two-site immunoenzymetric assay (Cygnus Technologies, Massachusetts, USA)., CXCL10 and CCL2 were measured in paired CSF and serum samples in the laboratory of Dr. Scarpini (Milan, Italy) by enzyme linked immuno absorbent assay, as previously described (Scarpini, 2002).

MRI analysis

Brain MR imaging was performed using a 1.5 T system (Siemens AG, Erlangen, Germany) as previously been described (Eikelenboom et al., 2003).
Two ratios of atrophy were calculated the parenchymal fraction (PF) defined as whole brain parenchyma/ intracranial volume and the ventricular fraction (VF) defined as ventricular volume/ whole brain parenchyma. The total volume of hyperintense lesions seen on the T2 images and of gadolinium enhancing lesions and hypointense lesions seen on T1 weighted images were calculated. In two subjects no adequate MRI data could be obtained.

Statistical analysis

For comparison of more than two groups, one-way ANOVA and Kruskal Wallis test were used as appropriated depending on the distribution of the data. For the correlations we used Pearson for normally distributed data and Spearman if the data were skewed. A level of 5% significance was employed.

Results

The CSF and serum concentrations of the chemokines CXCL10 and CCL2 are summarized in **Table 1**. In 7 CSF samples CXCL10 and in 1 CSF sample CCL2 was outside the detection limit of the assay. The CSF concentrations of CXCL10 and CCL2 were higher in CSF than in serum, but these differences did not reach the level of significance. In CSF CXCL10 correlated negatively with CCL 2 ($r=-0.33$; $p<0.05$; **Figure 1A**), in serum no correlation was found between the chemokines. The CSF leucocyte count was positively related to CSF CXCL10 ($r=0.34$; $p<0.05$; **Figure 1B**). No correlations could be shown between the IgG index and the chemokines. When the subgroups (RR, SP and PP) were compared to each other, no significant difference could be revealed. Also the clinical (EDSS, disease duration, age and time since last relapse) and MRI data (T2 and T1 hypointense and Gadolinium enhancing lesion load and the athrophy measurements, VF and PF) showed no significant correlations with the chemokine levels.

Table 1.

CCL2 and CXCL10 levels expressed as median values (interquartile range)

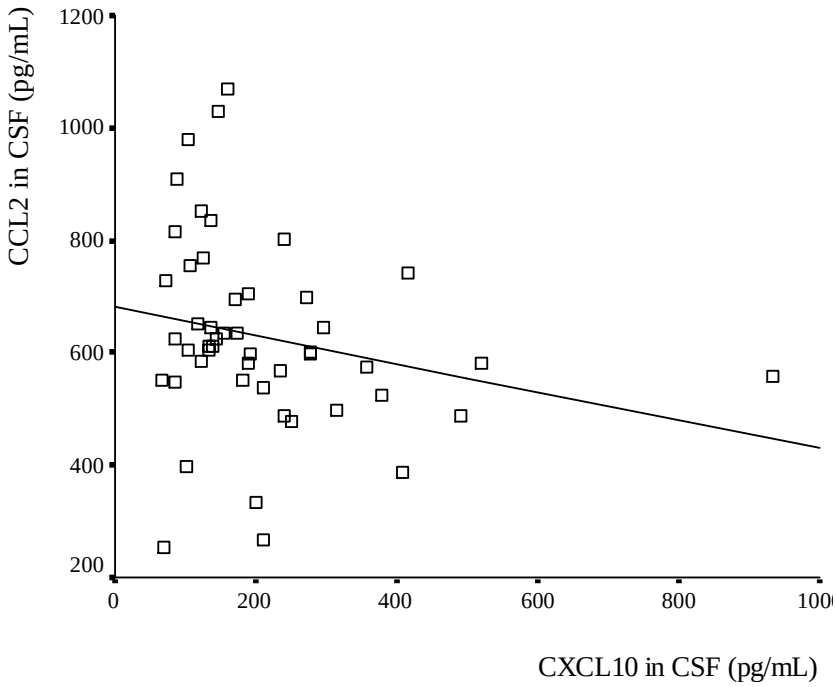
	CCL2CSF (pg/mL)	CCL2 serum (pg/mL)	CXCL10 CSF (pg/mL)	CXCL10 serum (pg/mL)
RR-MS (n=20)	601 (255-757)	325 (217-461)	136 (92-197)	111 (64.3-218)
SP-MS (n=21)	626 (570-720)	322 (258-481)	172 (107-276)	109 (75.5-241)
PP-MS (n=10)	599 (517-702)	328 (251-508)	225 (160-298)	125 (67-182)

RR=relapsing remitting, SP=secondary progressive, PP=primary progressive; n=number of subjects

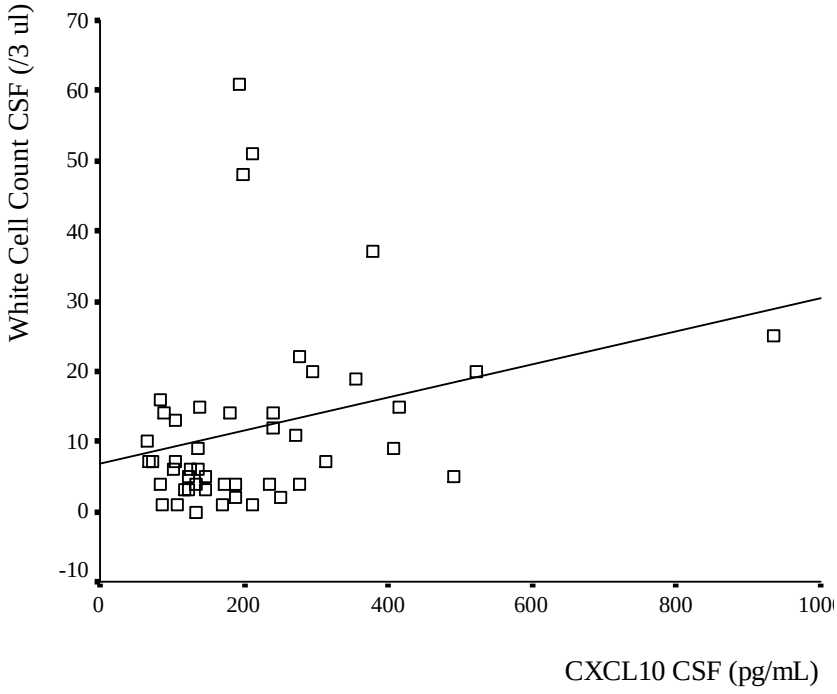
Figure 1.

CXCL10 correlations with WCC and CCL2

A. CSF CXCL10 v CCL2 (r=-0.33; p<0.05)



B. CSF CXCL10 v WCC concentration (r=0.34; p<0.05)



Discussion

This study was performed to test whether the chemokines CCL2 and CXCL10 play a role in multiple scleroses measured by clinical scales and MRI parameters of disease activity and state. CCL2 have been found to be strongly positive in chronic and active MS lesions (McManus et al., 1998). Therefore one would aspect that T1 (reflecting chronic lesions with axonal loss) and above all T2 lesion load (chronic and active lesions) would correlate with CCL2 measured in CSF, a body fluid most closely related to pathological processes in the brain. Also previously, differences in chemokine level CXCL10 were shown between relapsing onset MS and primary progressive MS (Scarpini et al., 2002). To test these before mentioned hypotheses we used samples from different patients, who were extensively studied (MRI scan, neurological examination) and analyzed these in the same laboratory (dr Scarpini).
We found in our samples an association between CXCL10 and CCL2, in CSF high CXCL10 was associated with low CCL2 and the white cell count was positively correlated with CXCL10 in CSF. When looked at other measures of disease activity and the chemokines, no correlation with IgG index or gadolinium-enhancing lesions could be revealed. Neither did we found differences between the subtypes.
These findings are in accordance with Sorensen et al. (2001) who also reported a significant positive correlation between CSF CXCL10 and leucocyte count. A few other studies have looked at gadolinium enhancement on MRI and chemokine levels in CSF and in line with our observations, no correlations could be revealed between active MRI lesions and CXCL10 or CCL2 (Sindern et al., 2002; Sorensen et al., 2001), although higher concentrations of chemokine receptor CXCR3 on T cells in CSF were seen in patients with gadolinium enhancing lesions (Sindern et al. 2002). Limited studies are known about the differences between MS subtypes and their chemokine levels.
Although earlier studies showed that in CSF CCL2 was lower and CXCL10 was significantly higher in MS compared to controls (Scarpini et al., 2002; Franciotta et al., 2001) most studies found these difference especially during relapses (Sorensen et al., 1999; Mahad et al., 2002, Bartosik-Psujek et al., 2005). The fact that we could not reveal such significant difference between the subtypes might be due to the selection of patients. In this study most patients in the RR phase were in a stable phase of the

disease based on clinical ground and MRI activity (gadolinium enhancement). This has the advantage that the chemokine concentrations are measured outside the acute phase of the disease, in which a lot of markers in MS are up regulated. Less is known about markers in the more stable phase of the disease. On the other hand, the composition of CSF reflects the extracellular interstitial fluid of the white matter (Sorensen et al., 1999) and chemokines may already be caught by their agonist and therefore no longer detectable in the CSF.
To our knowledge no other study has been undertaken to clarify the relation of these chemokine levels with markers of disease progression, as reflected on MRI by T1 and T2 lesion load and measures of atrophy, ventricular and parenchymal fraction.

In conclusion, our study suggests that in clinical stable MS patients CXCL10 and CCL2 measured in blood or serum should not be seen as markers of disease state, reflected by clinical and MRI measurements, nor can it discriminate between the subtypes.

Chapter 2.3

Expression of adhesion molecules on peripheral lymphocytes predicts lesion development in multiple sclerosis

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Abstract

The expression of adhesion molecules (alpha4beta1-integrin, LFA-1, ICAM-1) on T cells, measured by flow cytometry, was compared in different subtypes of multiple sclerosis (MS) and related to future lesion development as seen as delta T1 and T2 lesion load per year on magnetic resonance imaging (MRI). LFA-1 and alpha4beta1-integrin showed higher expression on CD4 and CD8 T lymphocytes in the secondary progressive compared to the relapsing remitting (CD4: $p<0.01$, p=ns, $p<0.05$; CD8: $p<0.001$, $p<0.001$, $p<0.001$, respectively) and primary progressive MS phase (CD4: $p<0.001$, $p<0.01$, $p<0.05$: CD8: $p<0.01$, $p<0.01$, $p<0.001$, respectively). The adhesion molecule expression of alpha4- ($r=0.31$; $p<0.05$) and beta1-integrin ($r=0.38$, $p<0.01$) on
CD4+ cells and LFA-1beta on both CD4+ and CD8+ ($r=0.28$, $p<0.05$ and $r=0.29$, $p<0.05$, respectively) cells was significantly related to increase in T2 lesion load. Our study provides further evidence for the involvement of integrins in lesion development, shown as T2 lesions on MRI in MS.

Introduction

Multiple sclerosis is a neurological disease characterized by multifocal areas of inflammation within the central nervous system (CNS). The binding of circulating autoreactive T cells and macrophages to the CNS endothelial cells and subsequent migration through the Blood-brain-barrier (BBB) is an essential step in the initiation of brain inflammation. This step of immune cell entry into the site of inflammation involves a complex cascade of events mediated by adhesion molecules (Canella et al., 1990; Springer, 1990). Even though the exact mechanism by which activated lymphocytes cross the BBB remains unclear, there is quite some evidence that transendothelial migration of leucocytes is thought to involve several independent, specialized adhesion pathways that are mediated by various classes of homing receptors and their vascular addressins. Adhesion molecules involved in immune responses have been classified into three families according to their structure: selectins, the immunoglobulin (Ig) superfamily, and integrins. In MS, adhesion molecules of these families are expressed on brain microvessel endothelial cells in active lesions of MS brain (Sobel et al., 1990; Washington et al., 1994; Bo et al., 1996). Selectins have been involved in the primary step of leukocyte migration and therefore they only may play a role in the very early adhesion process. Integrins and immunoglobulin superfamily members can be involved in primary as well as secondary (firm) adhesion of leucocytes (Springer, 1990; Chang et al., 2000). Immunoglobulin can function as ligand for integrins. Integrins are a family of transmembrane adhesion molecules that profoundly influence a variety of cell biological processes involved in cell-cell and cell-extracellular matrix communication. They consist of two groups, β1- and β2 intergrins. The presence of a variety of integrins on mono-nuclear cells and the upregulation of integrin ligands on endothelial cells during active disease points to a common pathogenetic role of integrins in experimental autoimmune encephalomyelitis (EAE). This notion was reinforced by therapeutic manipulation with monoclonal antibodies. From all published evidence, especially VLA-4 seems strongly involved in transendothelial migration of T cells in EAE, and the most promising target candidate for therapeutic interventions in MS (Yednock et al., 1992; Soilun-Hanninnen et al., 1997, Miller et al., 2003).

During the last few years at least two adhesion molecule pathways have been very well defined: the intercellular adhesion molecule 1 (ICAM-1; CD54) on endothelial cells and leukocytes together with its ligand on leukocytes, the lymphocyte function associated antigen (LFA-1, integrin alphaLbeta2; CD11a/CD18), and the vascular adhesion molecule 1 (VCAM-1) on endothelial cells and macrophages and its ligand on monocytes and lymphocytes, the very late antigen antigen 4 (VLA-4, alpha4beta1 integrin; CD49d/CD29). So far, correlations between adhesion molecules and MS disease status have mainly been investigated for serum and cerebrospinal fluid (CSF) concentrations of soluble forms of adhesion molecules. Even though no clear patterns have emerged yet, significant differences between different MS subsets were found and significant relations with active lesions on magnetic resonance imaging (MRI) have been reported (Giovannoni et al., 1997; Rieckmann et al., 1997; Khoury et al., 1999). Longitudinal studies investigating the predictive value of adhesion molecules with respect to the future course of the disease have not been performed. Recent MS trials have suggested additional evidence for the important role of adhesion molecules. In a large phase II study a humanized monoclonal antadhesion molecule antibody, Natalizumab, significantly reduced the frequency of new gadolinium enhancing lesion and relapses (Miller et al., 2003).

The goal of the present study was to investigate whether expression of adhesion molecules on T lymphocytes differs between MS subtypes and predicts disease activity with respect to future lesion development. The study is an extension on our previous study on chemokine receptors on T cells and their involvement on lesion load development (Eikelenboom et al., 2002). The chosen adhesion molecules in the present study represent the families involved mainly in the secondary step of leukocyte migration

(β1- integrin: alpha4beta1-integrin, β2- integrin: LFA-1 and immunoglobulin super family: ICAM-1).

Methods

Subjects

MS patients from the outpatient MS clinic at the VU Medical Centre were studied. The patients (n=124) participated in different treatment trials or natural history studies in our department from 1996 till 2000 (Eikelenboom et al., 2000). Blood samples were taken at the start of these studies. Using the classification system by Lublin and Reingold (1996), patients were assigned to the following subtypes: relapsing remitting (RR; n=39), secondary progressive (SP; n=40) and primary progressive (PP; n=45) MS. Demographic and patient characteristics are shown in **Table 1**. The subjects did not receive any inter-feron or corticosteroid treatment in the two months before the vena puncture. In the
30 days before the time of the blood sampling, no clinically relevant infections or relapses were ongoing.

Table 1.

Demographic characteristic of MS subjects (Mean ± SD)

	All (n=124)	RR (n=39)	SP (n=40)	PP (n=45)
M:F	56:68	12:27	26:14	18:27
Age (years)	48.9 ± 11.4	39.9 ± 6.9 [a]	49.3 ± 8.0 [a, b]	56.6 ± 11.5 [a, b]
EDSS	4.3 ± 2.0	2.2 ± 1.3 [a]	4.9 ± 1.1 [a, c]	5.6 ± 1.7 [a, c]
Disease duration (years)	10.2 ± 6.5	6.3 ± 5.0 [a]	12.4 ± 6.7 [a]	11.8 ± 5.9 [a]

M=male; F=female; RR=relapsing–remitting; SP=secondary progressive; PP=primary progressive; n=number of subjects

a) difference between RR MS and SP MS; RR MS and PP MS, $p<0.001$
b) difference between SP MS and PP MS, $p<0.001$
c) difference between SP MS and PP MS, $p<0.05$

MRI analysis

Sixty-nine patients of these patients (21 RR, 25 SP, 23 PP) have undergone at least two MRI scans with an interval of several years, without being treated with drugs that have been shown to modify the disease. While part of our patients participated in clinical trials, patients who did receive disease-modulating medicine were excluded from this analysis. Due to technical problems, of the 69 patients who had an MRI and were followed, 6 did not have a second T1 weighted image. Demographic data of these patients are listed in **Table 2**.

The subjects were scanned on a 1.0 or 1.5 T MRI system (Siemens AG, Erlangen, Germany); T2 (range: repetition time (TR) = 2000-3000 ms, echo time (TE) = 20-50 and 60-100, number of excitations (NEX) = 1) and T1 weighted images (TR = 600 to 700 ms; TE = 15 to 20 ms; NEX = 2) were made. Since the patients participated in different studies, the slice thickness varied slightly (3-5 mm). This was corrected for by calculating the volume of the lesions (area multiplied by the inter slice distance) (Kalkers et al., 2001). Lesion load measurements were performed on a work-station (Sun, Mountain view, California, USA) using semi-automated seed-growing software developed in house, based on local threshold. The total volume of hyper-intense lesions on the T2 images and of hypo-intense lesions seen on the T1 weighted images was calculated. The median follow up between the two MRI scans was 2.0 years (range: 0.5-5.5 years) and a delta lesion load per year was calculated, to correct for differences in the duration of follow up. All raters were masked for the clinical characteristics and the laboratory results of the subjects. They were well trained in measuring lesion loads with a coefficient of variation of less than 3.0 %.

Blood samples and laboratory analysis

Venous blood was collected in evacuated blood collection tubes (Vacutainer, Becton Dickinson, Meylan, France) containing sodium heparin. The samples were kept at room temperature and processed within 24 h. Peripheral blood-derived mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll-Isopaque density gradient centrifugation, and cryopreserved immediately. Freshly thawed and properly washed PBMC were incubated on ice with CD4-APC (Caltag, Burlingame, CA, USA) CD8-PerCP (Becton Dickinson, San Jose, CA, USA), in combination with adhesion markers CD54 (ICAM-1), CD11a (LFA-1alpha), CD18 (LFA-1beta) and CD29

Table 2.

Demographic and MRI characteristics in 69 MS subjects in whom MRI was available expressed as median change per year (interquartile range)

	All subjects (n=69 T2; n=63 T1)	RR (n=21 T2; n=17 T1)	SP (n=25 T2; n=23 T1)	PP (n=23 T1 and T2)
M:F	30:39	6:15	11:14	10:13
Age (years)	46.8 (41.6–56.2)	42.2 (35.4–44.3)	47.3 (44.0–55.6)	56.0 (48.8–62.6)
EDSS	4.0 (3.0–6.0)	2.0 (1–3.5)	4.5 (4.0–6.0)	6.0 (4.0–6.5)
Disease duration (years)	13.0 (10.0–18.0)	10.0 (8.0–12.5)	18.1 (12.1–22.0)	14.7 (11.4–16.0)
ΔT2LL/yr (cc)	0.22 (–0.13–1.5)	0.12 (-0.18–1.2)	0.59 (-0.23–3.3)	0.20 (-0.08–0.56)
ΔT1LL/yr (cc)	0.06 (0.0–0.39)	0.03 (-0.01–0.14)	0.40 (0.02–0.86)	0.03 (0–0.2)

M=male; F=female; RR=relapsing–remitting; SP=secondary progressive; PP=primary progressive; n=number of subjects; LL=lesion load; yr=year; Δ =delta.

(beta1-integrin; Sanquin, Amsterdam, The Netherlands) and CD49d (alpha4-integrin; Cymbus, Hampshire, UK) during 30 min. CD11a en CD49d and CD29 were Fitc labeled. CD54 and CD18 were coloured with PE labelled goat anti-Mouse IgG (Immunotech, CA, USA). Adhesion molecules expression was measured on CD4+ and CD8+ T-cells by flow cytometry (FacsCalibur). The adhesion molecules were expressed as mean fluorescence (mfl) in CD4+ and CD8+ T-cells.

Statistical analysis

Results are presented as mean and standard error of the mean (S.E.M.). One-way ANOVA and Kruskal Wallis test were used as appropriate. For the correlations the Spearman and Pearson r-values are given depending on the distribution of the data. We used a level of significance of 5% throughout in this exploratory study.

Results

Demographics

As expected and as shown in **Table 1** significant differences could be shown with respect to age, EDSS and disease duration between the clinical subtypes in our study (RR vs. SP vs. PP). Notice the SP subgroup has more male than female patients, a selection bias potentially caused by the fact that quite some of these patients participated in clinical trials.

Adhesion molecules between subsets

Representative FACS plots of peripheral blood mononuclear cells from two MS patients are shown in **Figure 1**. The mean levels of fluorescence of the different adhesion molecules tested ar are shown in **Figure 2**. Alpha4-integrin in CD8+cells was significantly lower in RR compared to SP ($p<0.001$) and PP ($p<0.01$) patients. Beta1-integrin gave higher mean fluorescence in the SP patient group both in CD4+ as in CD8+ cells, when compared to the RR group (in CD4+ $p<0.05$ and CD8+ cells $p<0.001$) and the PP patients (in CD4+ $p<0.05$ and in CD8+ cells $p<0.001$). LFA-1alpha in CD4+cells is elevated in SP compared to RR ($p<0.01$) and PP ($p<0.001$) subjects. LFA-1alpha in CD8+ cells showed significant differences between RR and SP ($p<0.001$), RR and PP
($p <0.05$) and SP and PP ($p<0.01$) subtypes. LFA-1beta showed both in CD4+ and in CD8+ T cells significant differences between the SP and PP group ($p<0.01$).

Adhesion molecules and their correlation with demographics

In the CD8+ cells modest but significant correlations could be shown between the integrins (alpha4 and beta1, respectively) and age, EDSS and disease duration as shown in **Table 3**.

MRI measures

Table 2 shows the annualized change in T1 and T2 lesion load for the whole group of MS patients that underwent MRI and for the different subgroups. SP subjects had a higher delta T2 and T1 lesion load development per year, compared to both other groups.

Figure 1.

Representative FACS plots of peripheral blood mononuclear cells from MS patients. T lymphocytes (R1), CD8+CD29+ (R2) and CD4+CD29+ (R3) cells were subsequently gated. Upper panel of histograms shows CD29 FITC fluorescence on CD8+ (left) and CD4+ (right) T cells of a relapsing–remitting MS patient, whereas lower panel shows higher CD8+CD29 (left) and CD4+CD29 (right) expression of a secondary progressive patient

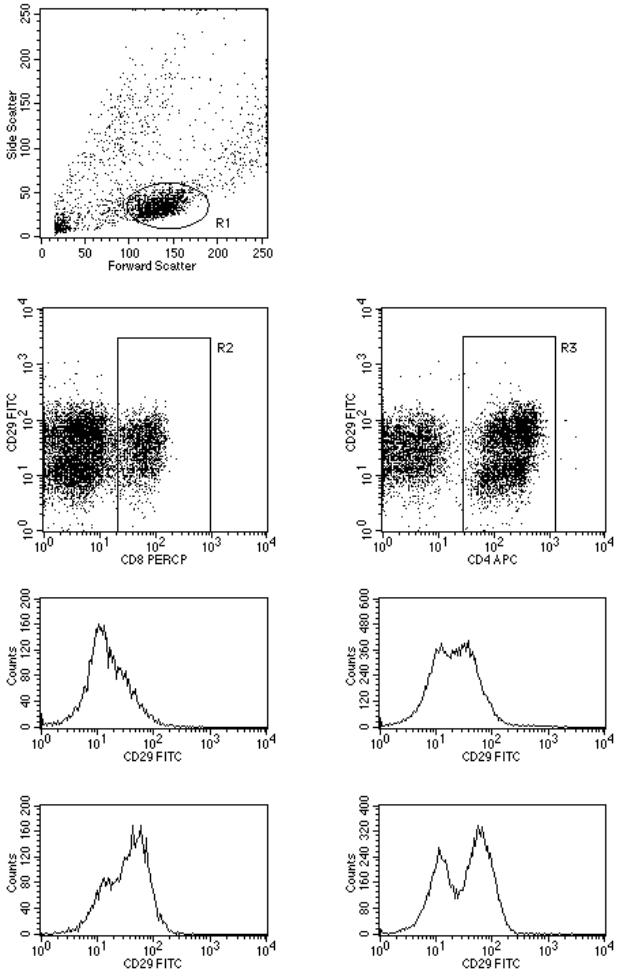

Figure 2.

Mean fluorescence intensity of adhesion molecule expressions on CD4+ and CD8+ lymphocytes in relapsing–remitting, secondary progressive and primary progressive MS patients. Results are expressed as mean values with S.E.M.

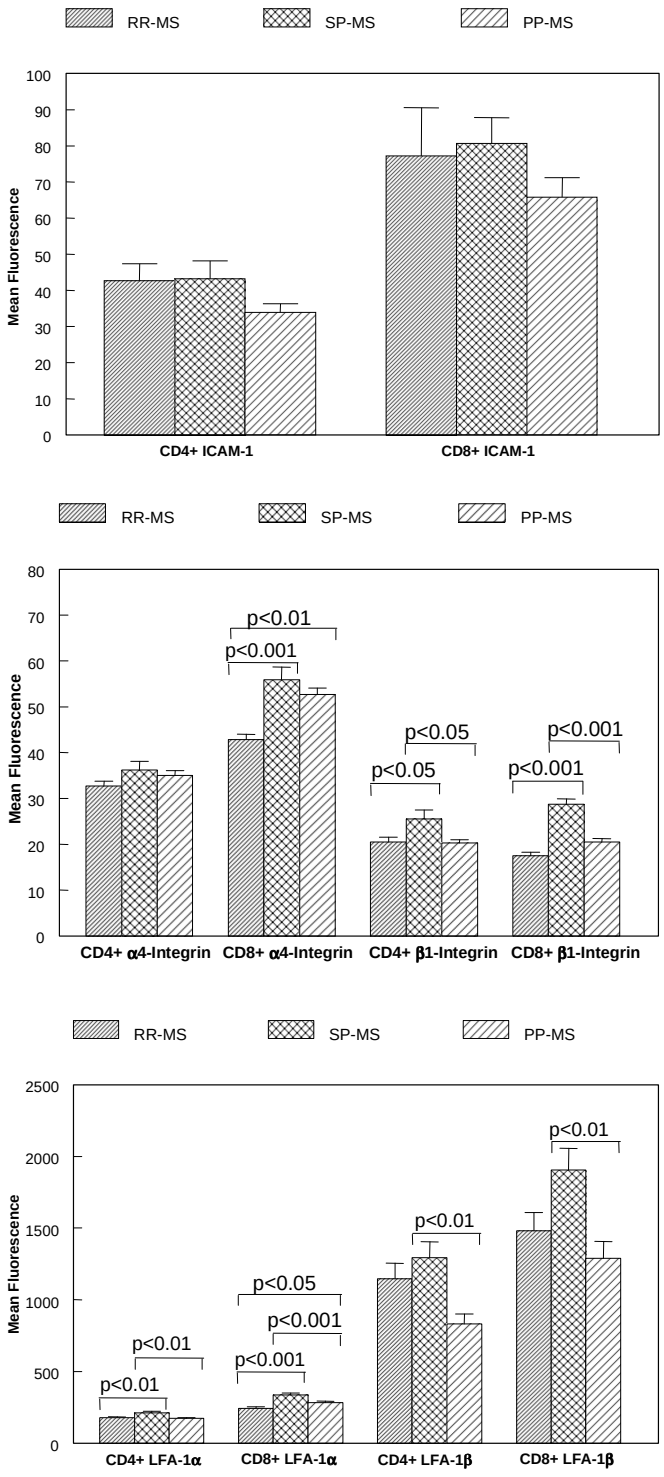

Table 3.

Correlations between demographic and MRI measurements and levels of cell surface bound adhesion molecules in blood of MS patients

Measurement studied	Age N=124	Disease duration N=124	EDSS N=124	ΔT2LL/yr (cc) N=69
ICAM-1 on CD4+ T cells	NC	NC	NC	NC
ICAM-1 on CD8+ T cells	NC	NC	NC	NC
LFA-1α on CD4+ T cells	NC	NC	NC	NC
LFA-1α on CD8+ T cells	NC	NC	NC	NC
LFA-1β on CD4+ T cells	NC	NC	NC	r=0.28 [a]
LFA-1β on CD8+ T cells	NC	NC	NC	r=0.29 [a]
α 4 integrin on CD4+ T cells	NC	NC	NC	r=0.31 [a]
α 4 integrin on CD8+ T cells	r=0.35 [c]	r=0.29 [b]	r=0.37 [c]	NC
β1 integrin on CD4+ T cells	NC	NC	NC	r=0.38 [b]
β1 integrin on CD8+ T cells	r=0.34 [c]	r=0.32 [b]	r=0.21 [b]	NC

Δ=delta; LL=lesion load; yr=year; cc= cubical centimetres; NC= not correlated; r=correlation coefficient.

a) $p<0.05$
b) $p<0.01$
c) $p<0.001$

Adhesion molecules and their correlation with MRI measures

Evaluating the whole group we found modest but significant correlations between the delta T2 lesion load and the following adhesion molecules: beta1-integrin in CD4+ cells (r=0.38; $p<0.01$), LFA-1beta in CD4+ cells (r=0.28, $p<0.05$) and CD8+ cells (r=0.29; $p<0.05$) and alpha4-integrin in CD4+ cells (r=0.31; pb0.05), as shown in **Table 3**. No significant relation was found with the delta T1 lesion load. Strikingly, in SP MS we found much higher correlations, again between the delta T2 lesion load and the LFA-

1beta in CD4+ and CD8+ cells ($r=0.54$, $p<0.01$; $r=0.50$, $p<0.05$, respectively) as well as the beta1-integrin in CD4+ cells ($r=0.76$, $p<0.05$). In the RR and PP MS subjects no significant correlations could be shown between adhesion molecules and the delta T2 lesion load. Looking at the delta T1 lesion load a significant correlation was only found with alpha4-integrin on CD4+ cells in RR MS patients ($r=0.51$; $p<0.05$). A sub-analysis was performed to look for differences between patients for whom MRI data were available versus those without MRI data. No differences were found between the two groups in their baseline immune data, age, EDSS or disease duration.

Discussion

The present study, investigating the expression of markers of ICAM-1, LFA-1 and VLA-4 in relation to MS subtype (RR vs. SP vs. PP) and to future lesion development on MRI of the brain, essentially is in line with previous studies that indicate that especially integrins are key molecules in the induction phase of lesion development in MS. A study on adhesion molecules on the surface of PBMC was also done to compare clinical subtypes of MS patients (Duran et al., 1999). In this study low levels of adhesion molecules (LFA-1alpha, integrin alpha4 and beta1 and ICAM-1) were found in SP and RR MS. Although our data also show decreased levels in RR MS, in the previous study PP MS showed higher expression than SP MS, which is in contrast with our findings. Also, our observation that LFA-1, and especially VLA-4, expression on both CD4 and CD8 T lymphocytes is elevated in the SP phase (compared to the RR phase) of the disease is new. We can only speculate that the subpopulation of SP patients in our study had more active disease (as was probably the case as judged on the increase in MRI lesion loads, presented in **Table 2**). In this context it might be appropriate to discuss the patients we studied in more depth. The RR patients included were selected for being untreated in a time period that interferon was already used for active patients. Therefore, our RR subjects might be less active; in line with this observation is the fact that there seems a minimal increase in the MR lesion load. However, many of the SP patients were selected at the onset of treatment trials, when disease-modifying treatment was not yet available for them, which most likely biased this group in the direction of being very active. Indeed, when compared to patients in large treatment trials in SP MS, our patients were relatively short in the SP phase of the disease and had marked increase on lesion load on the MRI. Consequently, the increased integrin expression seemed to be related to the (onset of the) active progressive phase of the disease. In addition, we saw correlations with demographic data (**Table 3**). We recognize the fact that correction for the different demographic data should be applied before concluding that differences between the MS subgroups are real. Statistical analyses, which appropriately correct for these parameters are difficult to establish. Obviously, RR and SP disease have different age and disease duration and it is difficult to decide whether these are confounders or determinants.

Our findings might represent the *in vivo* T correlate of the post-mortem observations in which a predominance of VCAM-1 and VLA-4 in chronic active MS lesions as compared to higher ICAM-1 expression in acute MS lesions was found (Cannella and Raine, 1995). In EAE adhesion molecule expression seems to precede the clinical symptoms and suggests a causal role of adhesion molecules in the initiation of CNS inflammation (Dopp et al., 1994). It might be the basis for our observation of significant, though moderate, correlations between integrin, especially VLA-4, expression and future lesion develop-ment on MRI, which, as far as we know, has not been reported previously. Recently, the expression of adhesion molecules on lymphocytes was studied in different subtypes of MS patients (Elovaara et al., 1998, 2000, Kraus et al., 2002). Up-regulated expression of VLA-4 and LFA-1 on immune cells in blood and CSF was demonstrated in patients undergoing active relapses as well as in relapsing patients after treatment with methylprednisolone (Elovaara et al., 1998, 2000). Up-regulation of these molecules in RR and SP MS is in line with our observations. From another perspective, however, the data presented by these authors are different, because they describe increased expression of VLA-4 and LFA-1 in RR patients compared to SP patients. These authors found moderate associations with lesion load measurements on MRI, just like we did on our longitudinal data. Strikingly, in another study (Kraus et al., 2002) the expression levels of cell surface bound adhesion molecules in peripheral blood was inversely correlated with parameters for sub-clinical disease severity and activity on cerebral MRI scans, suggesting that (subclinical) disease progression may be associated with a decrease of the expression of cell surface bound adhesion molecules on peripheral blood mononuclear cells, possibly as a result of migration of activated mononuclear cells into the CNS. Remarkably, when we looked at the progression on MRI, we could not confirm a significant inverse correl-ation between cICAM-1 and delta T2 or T1 lesion load in the whole group or the subgroups. Therefore, we conclude that cellular ICAM-1 should not be seen as a marker of disease progression, nor can it discriminate between the clinical subtypes (**Figure 2**).

A recent publication also showed that the soluble form of ICAM should not be seen as an indicator of MS activity long term (Flachenecker et al., 2002). Recently, correlations were shown between gadolinium enhancing lesions and beta1-integrin CD4+ helper inducer T cells (Wang et al., 2002). However, no correlations could be revealed between the T2 lesion load and the immuno-data in the before mentioned

cross-sectional study. The discrepancies between our results and other published data could, as discussed above, be explained by differences in patient selection or alternatively by differences in methods or timing of sampling in relation to disease activity. Since cross-sectional lesion loads or disease activity (gadolinium-enhancement) measures are affected by disease duration and by fluctuating disease activity, we tried to optimize correlations in our study by following patients longitudinally, which excludes inter-patient variability as a source of variation. Nevertheless, correlations were only modest, which from our perspective is not remark-able given the complex interactions involving multiple molecules that underlie the trans-migration of lymphocytes through the BBB, given the relatively high standard errors of integrin expression, as was also observed in other studies, and given the observation that changes in lesion load per year were relatively small. More importantly, it is generally recognized that in MS multiple pathophysiological processes are ongoing and that these processes are not uniformly represented across patient populations but can selectively predominate in individual patients. All the before mentioned reasons might weaken the correlations, rather than induce the relation found between the immunological and MRI markers. This *in vivo* relation seems to be supported by experimental and clinical studies and gives rise to believe that the findings indeed have biological meaning. In addition, we cannot fully exclude that the use of cryopreserved PBMC may have further weakened our correlations somewhat. However, viability and functional activity of cryopreserved cells have been extensively studied and it has been well established that PBMC maintain full functionality in assays if applied with experienced staff (Cavers et al., 2002; Kreher et al., 2003; Reimann et al., 2000; Weinberg et al., 2000). The aspect of our FACS plots (see also **Figure 1**) convincingly confirmed this. Furthermore, this method has several major advantages: such as combined analysis of multiple samples from different clinical groups, using numbered samples in random order under conditions completely blinded to clinical data.

The fact that our main correlations were found between integrins and increase in T2 lesion load rather than T1 lesion load might implicate that these adhesion molecules play a role in the initial development of new lesions rather than in the development of black holes that reflects more severe tissue destruction (van Waesberghe et al., 1999). Results from a phase II clinical trial in relapsing remitting MS applying a humanized antibody to the alpha4-chain further supports the important role of adhesion molecules. A

rather dramatic effect on the development of new active (gadolinium-enhancing) lesions was found (Miller et al., 2003), most likely as a result of a significant reduction in the adhesion and transmigration of leukocytes across the BBB. Furthermore, there might also be a role for integrins in the evolution from gadolinium enhancing lesions to the more destructive T1 hypointense lesions demonstrated by a suppressive effect of Natalizumab on this process (Dalton et al., 2004). In our study, we could only demon-strate correlations between the adhesion molecules and delta T2 lesion load and not T1 lesion load. This might be due to the relative small sample size or the fact that the changes per year of T2 lesions are higher than of hypointense T1 lesions. In addition, T2 lesions are histopathologically not specific and therefore reflect a whole range of pathological processes ongoing in the brain such as inflammation, edema, axonal loss and gliosis, whereas T1 hypointense lesions reflect axonal loss and enlargement of the extracellular space.

Recent evidence (Bitsch et al., 2000) suggests that especially CD8 positive lymphocytes might be crucial to development of disease progression as marked by axonal loss. Indeed, our data confirm an important role for CD8+ T cells in the differentiation of MS subgroups (shown in **Figure 2**) and LFA-1beta on CD8+ cells correlated significantly with the increase in T2 lesion load.

This study was an extension of a previous study (Eikelenboom et al., 2002), in which we analysed the predictive value of chemokine receptors on lesion development on MRI. We also analyzed whether the prediction of development of lesions could be increased by a combined analysis of chemokine receptor and adhesion molecule data. Even though this combined analysis showed some trends (lesion development in the whole patient group being especially predicted by the chemokine receptor CCR5 and by the above mentioned adhesion molecules in the SP patients, data not shown), it did not further strengthen our results nor did it give new insights. This might be due to the fact that the immuno data are also related to each other.

In our present study we were able to detect statistically significant differences between subgroups of patients as well as significant correlations with future lesion development on MRI. Although definite conclusions cannot be drawn and further studies are warranted, our observations may link integrin expression to future disease activity and disease prognosis and also lend support to an ongoing treatment program investigating the effect of therapeutic inhibition of alpha4-integrin mediated T cell migration in MS.

Chapter 2.4

Sex differences in pro-inflammatory cytokine profiles of progressive patients in multiple sclerosis

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Abstract

The objective of this article is to evaluate the presence of sex differences in expression of cytokines in both CD4+ and CD8+ T cells derived from peripheral blood of untreated multiple sclerosis (MS) patients.

The predominance of females in MS and other autoimmune diseases may be related to their differential responses in many immunological settings. Recent data show beneficial effect of sex hormones on pro-inflammatory cytokine levels and on MRI in MS. Better understanding of gender differences is warranted. In this study one hundred twenty four MS subjects (M: F; 56:68) and 34 healthy controls (M:F; 12:22) were included. Stimul-ated peripheral blood derived CD4+ and CD8+ T cells were analysed for IFN-gamma, IL-2, TNF-alpha, IL-4, IL-10 and IL-13 production. There were no significant differen-ces for these cytokines between male and female MS subjects in the whole group. Compared to males female patients had higher pro-inflammatory cytokine levels in the progressive phase of the disease and lower levels in the relapsing phase of the disease.

In conclusion, the data presented indicate that cytokine production, and sex differences in cytokine production might differ between disease phases, probably related to underlying disease mechanisms.

Introduction

Multiple Sclerosis (MS) is an autoimmune mediated disease of the central nervous system (CNS). Pathologically, inflammatory infiltrates contain T cells, monocytes and macrophages, leading to demyelination and axonal loss (Martino et al., 1999). One of the determinants in this immune response is thought to be the release of cytokines by T cells. Cytokines can be classified in pro-inflammatory cytokines, such as interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα) and interleukin-2 (IL2), produced by T helper (Th) 1 cells and anti-inflammatory cytokines, like interleukine-4 (IL-4) and interleukine-10 (IL10), which are produced by Th2 cells. A misbalance between Th1 and Th2 type cells is thought to be one of the hallmarks in MS and has been hypothesized in other autoimmune diseases as well (Charlton et al., 1995).
The predominance of females in MS (male: female ratio 1:2; Duquette et al., 1993) and other autoimmune diseases may be related to their differential responses in many immunological settings (Voskuhl et al., 2002; Whitacre et al., 1999; Whitacre et al., 2001). Another indication of the important role of sex hormones is the observation that the number of relapses is reduced during the third period of pregnancy and increased postpartum. Cytokine production seems to be altered by sex hormones both *in vitro* and *in vivo* (Gilmore et al., 1997; Verthelyi et al., 2000). In addition, it has been shown that sex hormones can affect the number and/or activation state of lymphocytes and that disease severity in patients with autoimmune disorders is influenced by serum concentrat-ions of estrogen, progesterone and/or androgen (Cutolo et al., 1988; Lahita et al., 1981). Although the precise mechanism is poorly understood, convincing evidence of the interaction between sex hormones and the immune system has recently been obtained by demonstrating that non-pregnant female MS patients who were treated with the pregnancy hormone estriol had a significant decrease in interferon-gamma levels in peripheral blood mononuclear cells associated with reductions in numbers and volumes of gadolinium enhancing lesions on monthly cerebral magnetic resonance images (Sicotte et al., 2002).
Recently, a gender bias towards pro-inflammatory Th1 responses to myelin proteins (high IFN gamma, no IL5 response) has been reported in 11 female MS patients compared to 11 male patients (Pelfrey et al., 2002). Sex differences in pro-inflammatory cytokines were also found in a small group of relapsing remitting MS

patients (Nguyen et al., 2003). Surprisingly, in this study men secreted higher pro-inflammatory cytokines than women. Previously, we have shown that cytokine production also differs in the separate phases of the disease (Killestein et al., 2003). The goal of the present study is to analyze the presence of sex differences in cytokine expression in a large cohort of untreated MS patients. Therefore we evaluated 34 healthy controls and 124 MS patients and looked for gender differences in pro- and anti- inflammatory cytokine expression in CD4+ and CD8+ T cells, in the MS subjects as a whole group as well as in the different clinical subtypes.

Methods

Subjects

Data of one hundred twenty-four MS patients with clinically definite MS and 34 healthy controls (HC) were reanalyzed (Killestein et al., 2001 and 2003) The HC consisted of 12 males and 22 females with a mean age of 43.1 (±13.5). In the MS subjects 39 had a relapsing remitting (RR) course, 40 patients were secondary progressive (SP) and 45 were primary progressive (PP). Demographic characteristics of the MS patients are given in **Table 1**. None of these patients received immuno-modulatory treatment or had a clinical relapse in the previous two months before blood sampling. None of the patients had a clinically relevant infection or other immunological, metabolic, neurological or cardio-vascular disease at the time of the vena puncture. None of the female patients were pregnant. Sixteen patients used oral contraceptives; they were all from the relapse onset subtype (13 RR and 3 SP). The Ethics Committee of the VU Medical Center approved the study and the participants gave informed consent.

Table 1.

Demographic data of male and female MS subjects in the different subtypes expressed as mean value with standard deviation

	RRMS		SPMS		PPMS	
	Male (n= 12)	Female (n= 27)	Male (n= 26)	Female (n= 14)	Male (n= 18)	Female (n= 27)
Age (years)	41.5±7.0	39.3±7.0	49.7 ±6.8	49.0±9.3	55.6±8.5	57.2±13.2
Disease duration (years)	10.1±2.6	11.7±5.5	19.1 ±6.4	17.2±6.7	15.5±7.3	16.5±4.7
EDSS	2.4±1.3	2.0 ±1.4	4.6±1.3	5.2±0.9	5.8±1.7	5.5±1.6

Blood samples and laboratory analysis

Venous blood was collected in evacuated blood collection tubes (Vacutainer, Becton Dickinson, Meylan, France) containing sodium heparin. Samples were kept at room

temperature and processed within 24 hr. Peripheral blood-derived mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll-Isopaque density gradient centrifugation, and cryopreserved immediately. Freshly thawed and properly washed PBMC were treated for 4 hours with Phorbol Myristate Acetate and Ionomycin in the presence of the protein-secretion inhibitor Monensin. After cell surface staining with CD4-APC and CD8-PerCP, cells were washed, fixated and permeabilized properly. Cytoplasm was stained with FITC labelled and PE labelled cytokine monoclonal antibodies. Cells were analysed applying four colour staining technique using Calibur Facsscan and Cellquest software as previously been described (Killestein et al., 2001). CD4+ and CD8+ T cells were gated and flow cytometry results were expressed as percentages of T cells producing Th1 type cytokines IFNγ, TNFα and IL2 and the Th2 type cytokines IL4, IL10 and IL13.

Statistical analysis

Depending on the distribution of the data independent sample *t* tests or Mann-Whitney U tests were performed to compare men and women within the HC and the MS patients, in the total group (with and without the female patients who used oral contraceptives), as well as the subgroups based on disease course. In addition, univariate analysis of variance was performed to correct for age, disease duration and EDSS. The level of significance was set at 0.05.

Results

There were no significant differences for the pro-inflammatory cytokines IFNγ, TNFα, IL2 or the anti-inflammatory cytokines IL4, IL10 and IL13 between males and females in the healthy controls (**Table 2**) or MS subjects when analyzed as the whole group (data not shown). Strikingly, there were sex differences related to the disease phase. In the progressive phase of the disease female patients had higher pro-inflammatory cytokine levels than males. In CD4+ T cells, this difference was seen in SP subjects for TNFα ($p=0.014$) and in PP subjects for all of the pro-inflammatory cytokines (IFNγ $p=0.017$, TNFα $p=0.03$, IL2 $p=0.013$) examined. Vice versa, in patients with relapsing remitting MS lower levels of pro-inflammatory cytokines were found in females, but these differences, though consistent, did not reach levels of statistical significance. No significant differences were found for anti-inflammatory cytokines IL 4, IL10 and IL13 in CD4+ cells (shown in **Table 2**). The same trend as described for CD4+ cells was also found for CD8+ T cells (**Table 2**), but only in RR patients IL2 in CD8+ cells was significantly raised in males compared to females. When we excluded the females (13 in the RRMS group), who used oral contra-ceptives, there was no significant difference ($p=0.06$) in the RR group in IL2 CD8 + cells between males (median value: 9.2; IQR 5.4-15) and females (5.1; IQR 2.9-10.2). After adjustment for age, disease duration and EDSS, sex differences in pro-and anti-inflammatory cytokines remained largely unchanged, although in RR MS adjusted IL2 in CD4+ cells was now significantly higher in men compared to women ($p< 0.01$).

Table 2

Percentage CD4+ or CD8+ T cells producing anti- or pro-inflammatory cytokines in male and female MS subjects in different subtypes expressed as median value with interquartile range

Cytokines	HC		RRMS		SPMS		PPMS	
Anti-inflammatory	Male	Female	Male	Female	Male	Female	Male	Female
IL4 CD4+	2.0 (1.0-3.0)	2.0 (2.0-3.0)	1.9 (1.3-3.7)	2.0 (1.3-3.1)	2.4 (1.6-4.0)	2.8 (2.0-4.1)	2.0 (1.2-3.8)	1.9 (1.0-3.9)
IL4 CD8+	3.0 (1.3-4.0)	2.0 (1.8-3.0)	1.0 (0.8-2.7)	1.1 (0.6-2.3)	2.1 (1.1-4.4)	1.6 (0.6-2.6)	3.0 (1.5-4.3)	2.9 (1.5-5.2)
IL10 CD4+	2.0 (1.5-4.0)	2.5 (2.0-4.8)	1.4 (1.0-2.9)	1.3 (0.8-2.0)	1.1 (0.7-2.0)	1.0 (0.7-1.9)	2.5 (1.5-4.0)	2.6 (1.8-3.2)
IL10 CD8+	1.0 (1.0-4.0)	3.0 (1.3-3.0)	1.0 (0.3-1.7)	0.7 (0.4-1.2)	0.5 (0.3-0.9)	0.6 (0.2-1.1)	1.9 (1.0-2.4)	2.3 (1.2-3.0)
IL13 CD4+	3.0 (2.0-5.0)	3.0 (2.0-4.0)	2.3 (1.3-4.6)	2.3 (1.4-3.5)	2.5 (2.1-3.6)	2.9 (2.1-3.7)	0.8 (0.4-1.6)	1.4 (0.6-2.0)
IL13 CD8+	3.5 (1.5-4.8)	4.0 (2.0-6.0)	0.6 (0.6-2.3)	1.0 (0.6-2.2)	1.7 (1.0-4.8)	1.6 (0.8-3.1)	0.6 (1.5-2.9)	1.8 (1.0-2.9)
Pro-inflammatory								
IL2 CD4+	21 (17-24)	23 (17-33)	28 (26-39)	26 (17-34)	27 (17-34)	29 (21-36)	6.5 (4.5-20)*	20 (10-26)*
IL2 CD8+	14 (11-19)	11 (7.0-13)	9.2 (5.4-15)*	6.0 (3.0-12)*	9.0 (4.8-15)	6.1 (4.1-10)	6.6 (3.3-9.1)	6.4 (5.0-11)
IFN γ CD4+	9.7 (5.3-12)	10 (7.0-13)	18 (11-21)	13 (10-19)	13 (8.5-19)	19 (12-26)*	6.4 (4.6-11)*	14 (6.3-18)*
IFN γ CD8+	20 (12-22)	20 (12-29)	29 (20-41)	23 (14-33)	31 (20-43)	34 (25-42)	16 (6.8-26)	21 (15-31)
TNF α CD4+	14 (11-29)	22 (16-25)	23 (16-28)	19 (14-28)	19 (12-26)*	26 (17-37)*	6.5 (3.2-13)*	12 (7.2-22)*
TNF α CD8+	11 (8.0-20)	15 (6.8-16)	7.5 (5.0-11)	5.9 (3.0-9.7)	8.8 (5.8-12)	12 (5.4-18)	4.3 (2.2-10)	6.6 (3.0-8.4)

* Differences between males and females, $p<0.05$

Discussion

This is the first large study reporting sex differences in cytokine producing T cells of MS patients. Although T cell cytokine profiles did not differ between sexes in the healthy controls or total MS group, we found significant differences between subgroups. In RR patients, pro-inflammatory cytokine production is higher in males than in females and vice versa, during the progressive phase of the disease (SP and PP) levels are higher in females compared to males. Strikingly, this pattern between males and females in relation to disease phase seems to be very consistent for all three pro-inflammatory cytokines tested. These observations provide additional support to the hypothesis that cytokine production (Killestein et al., 2001) and sex differences in cytokine production in MS are disease phase related. This may be associated with the specific mechanisms underlying disease phases, these being mainly inflammation in the relapsing phase and degeneration in the progressive phase of the disease. A previous study in only 22 MS patients revealed a significant immune response sex difference (Pelfrey et al., 2002), suggesting Th1 skewing in female MS patients, thus pointing in the same direction as our study in progressive patients. These authors also found no gender difference with respect to the Th2 cytokine they tested, IL5. Our results, though not all statistically significant, are also in accordance with the findings of another study, which showed higher percentages of TNFα in men in RR MS patients (Nguyen et al., 2003).

It is difficult to understand our observations in terms of the underlying pathophysiology of the disease since they can theoretically be involved in relevant pathomechanisms, but also only represent consequences of the underlying pathological process. In this context, one might speculate on relations of our observations with recent evidence that suggests that inflammation can also be beneficial and can even have neuroprotective effects (Kerschensteiner et al., 2003). In our study females in the progressive phase of the disease have higher pro-inflammatory cytokines than males. The concept of "neuroprotective immunity" might explain why in women the disease course is often milder and less disabling than in men. This concept is supported by a recent MRI study, in which men demonstrated fewer contrast-enhancing lesions, but a higher proportion of lesions evolving into 'black holes' compared with women, indicating that men with MS are more prone to

develop more destructive and less inflammatory lesions than women (Pozzilli et al., 2003). Recently, the same group (Tomassini et al., 2003) presented another study of gender-related modulation of pathological changes in RR MS being in opposite direction for males versus females. The authors studied serum sex hormone levels and related these to MRI characteristics of brain lesions in MS. Greater brain damage as documented by T1-hypointense lesion load was associated with a higher testosteron/ oestradiol ratio in women, but with a lower testosteron/oestradiol ratio in men, indicating that sex hormones (both estradiol and testosterone) are involved in the process leading to irreversible tissue damage, but that their role might differ between sexes.

Of course, we must be cautious in interpreting the data of our study; although we were able to show consistent and partially significant differences between males and females, their level of significance was low. In addition, the male:female ratios are not fully representative for a MS population. This is due to the fact that it was an extension of a previous study. We do hope, however, that our study, suggesting that sex differences in cytokine production are disease phase related, provides a challenge for future studies.

Chapter 2.5

Opticospinal multiple sclerosis:

a pathogenetically distinct form?

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Abstract

Opticospinal multiple sclerosis (OSMS) seems to be a clinical subform of MS, with distinct features, resembling Neuromyelitis Optica (NMO). Pathologically NMO seems to differ from MS. To further investigate the difference between OSMS and CMS we re-analyzed existing immunodata on peripheral T cells on 124 MS patients, in which 11 patients met the criteria for OSMS. We found that OSMS compared to classic MS tend to have higher disability after shorter disease duration and immunological parameters provide evidence for a more Th2 mediated disease, resembling NMO.

Introduction

Multiple Sclerosis (MS) is the prototypic idiopathic inflammatory demyelinating disease of the Central Nervous System (CNS), with different clinical forms as defined by Lublin and Reingold (1996). There is recent concensus that one subtype of relapsing-remitting demyelinating disease, Neuromyelitis optica (NMO, Devic's disease), should be recognized as a separate entity. Clinically, the principal distinguishing feature of NMO is that the neurological findings are essentially restricted to the optic nerve and spinal cord, both at the onset and through follow up, which is most unusual for MS, where cognitive, brainstem and cerebellar involvement are commonplace. Other features that seem to distinguish NMO from prototypic MS are poor outcome of attacks, MRI findings (normal or non-specific findings in the brain, lesions in spinal cord often extending over multiple segments) and typical cerebrospinal fluid (CSF) findings (pleiocytosis in absence of oligoclonal IgG) (Wingerchuk et al., 1999; Weinshenker et al., 2003). Pathologically, NMO seems to differ from prototypic MS because in lesions macrophages, eosinophils, complement activation, vascular fibrosis and hyalinization are more prominent, pointing to a role for humoral immunity (Lucchinetti et al., 2002). As such, NMO is probably the clinical correlate of the type of lesions characterized as type II by Lassmann et al. (2001). We hypothesized that prototypic MS and NMO represent the extremes of a continuum rather that being fundamentally different diseases. Inline with this hypothesis we assumed that within the spectrum of human inflammatory demyelinating disease there would be an subgroup of patients with opticospinal MS (OSMS) as an intermediate entity. OSMS has been described previously; it is the most frequently occurring MS phenotype in Asia. Clinically and pathologically it clearly seems to be intermediate between prototypic MS and NMO, and therefore we were surprised to read recent reports that suggest that immunologically it is not. Kira (2003) reported a shift towards a Th1 T lymphocyte response, even more consistently present than in non-opticospinal, prototypic MS, thereby positioning it at quite a distance from NMO, which is characterized by a B-lymphocyte and Th2-lymphocyte response (Lucchinetti et al., 2002).

Methods

We reanalyzed existing data on 124 Caucasian patients with definite MS who had participated in studies where flow cytometric measurements of Th1 cytokines (IFN gamma, IL-2, TNF-alpha), chemokines (CXCR3 and CCR5) and Th2 cytokines (Il-4, IL-10 and IL-13) has been performed (Eikelenboom et al., 2002; Killestein et al., 2001). For this study we defined OSMS as MS with clinical evidence of only optic and nerve and spinal cord involvement, at least one clinical relapse, and a brain MRI not typical for MS, in that it showed only few or atypical lesions, accorinding to Kira (2003). For the 124 MS patients, 11 (8.8%) fulfilled our criteria for OSMS. There were no differences in the male versus female ratio between OSMS and prototypic MS patients. Compared with prototypic MS patients, OSMS patients tended to have higher disability (mean EDSS 6.0 versus 4.0, with corresponding IQR from 4 to 7 and from 2.5 to 6, respectively) after similar or even shorter disease duration (means of 13.0 versus 14.6 years). Nine of these patients were in the progressive phase of the disease; there was no evidence of mainly relapse-derived accumulation of disability in most patients.

Results

Table 1 shows cytokine and chemokine receptor immunodata on CD4 and CD8+ peripheral T cells in the different groups. Strikingly, in OSMS a lower percentage of the Th1 cytokine IFN-gamma was found in CD4+ T cells as well as a higher percentage of the Th2 cytokine IL-10 in CD8+ T cells compared to prototypic MS. No differences were shown between the two groups for the chemokine receptors CXCR3 and CCR5.

Table 1.

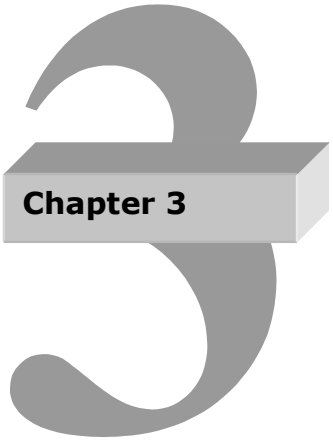
Median percentage of cytokine producing and chemokine receptor expressive CD4+ and CD8+ T cells in OSMS versus prototypic MS (interquartile range)

			Prototypic MS (n=113)	OSMS(n=11)
Th1	IFNγ	CD4+	13.5 (9.1-19.0) [a]	9.6 (5.0-13.6) [a]
		CD8+	23.8 (15.7-36.5)	21.1 (14.0-31.6)
	TNFα	CD4+	17.2 (10.9-26.3)	15.5 (4.7-21.0)
		CD8+	6.8 (4.0-11.0)	7.1 (3.0-11.1)
	IL2	CD4+	24.9 (15.4-32.2)	18.6 (10.5-24.6)
		CD8+	7.2 (4.1-11.3)	7.5 (4.9-14.1)
	CXCR3	CD4+	22.0 (15.0-30.0)	23.0 (15.0-30.0)
		CD8+	43.0 (30.0-67.0)	48.0 (26.0-60.0)
	CCR5	CD4+	12.0 (9.0-19.0)	12.0 (9.0-15.0)
		CD8+	32.0 (20.0-45.0)	31.0 (20.0-40.0)
Th2	IL4	CD4+	2.3 (1.4-3.8)	2.0 (1.3-3.1)
		CD8+	1.8 (0.9-3.5)	1.7 (1.4-5.9)
	IL10	CD4+	1.5 (0.9-2.5)	2.8 (1.8-3.6)
		CD8+	0.9 (0.4-1.8)[a]	2.1 (1.0-2.5) [a]
	IL13	CD4+	2.3 (1.1-3.3)	1.0 (0.70-3.1)
		CD8+	1.4 (0.8-2.9)	1.1 (0.9-5.5)

a) level of significance $p<0.05$

Discussion

Our data show that OSMS occurs at a frequency of about 10% in Caucasians, that clinical characteristics are similar to those reported for Asian OSMS (higher disability following shorter disease duration), but that immunological parameters differ in that they provide evidence for Th2 mediated disease rather than for Th1 mediated disease, very comparable with NMO. As such (Western) OSMS can indeed be postioned between prototypic MS and NMO, thereby providing support for the hypothesis that inflammatory demyelinating disease pathogentically represents a spectrum rather than qualitatively different entities. It can be speculated that this spectrum is associated to the spectrum of immunopathological abnormalities recently identified in inflammatory demyelinating disease (Lassmann et al., 2001). Wheras prototypic MS might correspond with a type I 'Lucchinetti pattern' and NMO to a type II pattern, OSMS probably should be positioned in between. These data provide preliminary thoughts on correlation between the current pathological classification of the disease and clinical phenotype; whether these thoughts will ultimately be incorporated in currently applied clinical classification schemes will only be known when more data become available.



Biomarkers reflecting pathology of the central nervous system

Chapter 3.1

Markers for different glial cell responses in multiple sclerosis: clinical and pathological correlations

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Abstract

Disease progression in multiple sclerosis occurs within the interface of glial activation and gliosis. This study aimed to investigate the relationship between biomarkers of different glial cell responses: (i) to disease dynamics and the clinical subtypes of multiple sclerosis; (ii) to disability; and (iii) to cross-validate these findings in a post-mortem study. To address the first goal, 51 patients with multiple sclerosis [20 relapsing remitting (RR), 21 secondary progressive (SP) and 10 primary progressive (PP)] and 51 neurological control patients were included. Disability was assessed using the ambulation index (AI), the Expanded Disability Status Scale score (EDSS) and the 9-hole PEG test (9HPT). Patients underwent lumbar puncture within 7 days of clinical assessment. Post-mortem brain tissue (12 multiple sclerosis and eight control patients) was classified histologically and adjacent sites were homogenized for protein analysis. S100B, ferritin and glial-fibrillary acidic protein (GFAP) were quantified in CSF and brain-tissue homogenate by ELISA (enzyme-linked immunosorbent assay) techniques developed in-house. There was a significant trend for increasing S100B levels from PP to SP to RR multiple sclerosis ($p < 0.05$) S100B was significantly higher in RR multiple sclerosis than in control patients ($p < 0.01$), whilst ferritin levels were significantly higher in SP multiple sclerosis than in control patients ($p < 0.01$). The S100B: ferritin ratio discriminated patients with RR multiple sclerosis from SP, PP or control patients ($p < 0.05$, $p < 0.01$ and $p < 0.01$, respectively). Multiple sclerosis patients with poor ambulation (AI ≥7) or severe disability (EDSS >6.5) had significantly higher CSF GFAP levels than less disabled multiple sclerosis or control patients ($p < 0.01$ and $p < 0.001$, respectively). There was a correlation between GFAP levels and ambulation in SP multiple sclerosis ($r = 0.57$, $p< 0.01$), and between S100B level and the 9HPT in PP multiple sclerosis patients ($r = –0.85$, $p < 0.01$). The post-mortem study showed significantly higher S100B levels in the acute than in the subacute plaques ($p < 0.01$), whilst ferritin levels were elevated in all multiple sclerosis lesion stages. Both GFAP and S100B levels were significantly higher in the cortex of multiple sclerosis than in control brain homogenate ($p < 0.001$ and $p< 0.05$, respectively). We found that S100B is a good marker for the relapsing phase of the disease (confirmed by post-mortem observation) as opposed to ferritin, which is elevated throughout the entire course. GFAP correlated with disability scales and may therefore be a marker for irreversible damage. The results of this study have broad implications for

finding new and sensitive outcome measures for treatment trials that aim to delay the development of disability. They may also be considered in future classifications of multiple sclerosis patients.

Introduction

In his remarkable 1868 papers, Charcot (1868) distinguished three steps in the pathology of the disease he first described, '*la sclérose en plaques*' (multiple sclerosis). (i) Initial astrocytic and microglial activation: '*la multiplication des noyaux et l'hyperplasie concomitante des fibres réticulées de la névroglie sont le fait initial*'; (ii) secondary neuro-axonal degeneration: '*l'atrophie dégénerative des éléments nerveux est secondaire*'; and (iii) astrogliosis: '*la névroglie fait place au tissu fibrillaire*', which he considered to represent the anatomical substrates of progressively impaired locomotor activity, '*est considérée à juste titre comme le substratum anatomique de l'ataxie locomotrice progressive*'.

Of these three steps, axonal damage has become one of the most intensely studied aspects of recent multiple sclerosis research. The clinical relevance of the glial response has, however, received less attention despite recent evidence that glial pathology can precede secondary axonal degeneration (Griffiths et al., 1998). During the glial response different cell-type-specific proteins are released, which can be measured in the CSF (Thompson and Green, 1998). The CSF concentration of these brain-specific proteins (BSP) depends upon the synthesis, catabolism and cellular integrity of astrocytes and microglia. We decided to analyse several BSP in the CSF of multiple sclerosis patients. The BSP chosen to be quantified in this study was S100B for astrocytic activation (Green et al., 1997 b), ferritin for microglial activation (Keir et al., 1993) and glial fibrillary acidic protein (GFAP) for astrogliosis (Rosengren et al., 1995). Astrocytic and microglial activation describe the immediate cellular response to any CNS challenge (Streit et al., 1988; Eng and Ghirnikar, 1994; Barron, 1995) and astrogliosis is defined as the fibrinoid scar that replaces lost tissue (Charcot, 1868; Eng et al., 1970). GFAP was first isolated from multiple sclerosis plaques and subsequently found in normal astrocytes (Eng et al., 1970). S100B has been used for many years as a marker for astrocytic proliferation. Interest in the role of S100B in neurological diseases has recently focused on its ability to exhibit both neuroprotective and neurotoxic properties (Donato, 2001). Ferritin has been widely

used by histologists for staining microglial cells. The designs of previous studies did not allow sufficiently detailed analysis of both disease subtype and disability in relation to CSF BSP levels (Rosengren et al., 1995; Jongen et al., 1997, 1998; LeVine et al., 1999). Consequently, most authors did not show any statistically significant difference between clinical subtypes or any direct correlation with disability. Confirmation of some results has been hampered by the use of tests that have only been available to the original laboratory (Rosengren et al., 1992, 1995). To overcome this problem we have developed a new ELISA (enzyme-linked immunosorbent assay) technique for quantification of GFAP using commercially available reagents.

This is the first study quantifying CSF levels of S100B, GFAP and ferritin in well defined clinical multiple sclerosis subtypes, which are not heavily biased by patients having acute relapses. This is important because release of biomarkers during the acute phase of disease is slanted towards relapse-related tissue destruction.

This cross-sectional study aimed to investigate the relationship between the concentration of biomarkers for glial reaction in the CSF with the clinical subtypes and the degree of disability in multiple sclerosis patients. Assumptions that CSF BSP levels are related to pathology in multiple sclerosis brains were tested in a post-mortem brain tissue study comparing multiple sclerosis with control brains.

The hypotheses underlying the study were: (i) CSF BSP levels are influenced by the dynamics of disease and can be used to distinguish different multiple sclerosis subtypes; (ii) CSF BSP levels relate to the degree of disability; and (iii) BSP levels are related to histopathological features of CNS lesions. Results from this study may be exploited to try to establish new outcome measures for treatment trials that aim to delay the development of disability in multiple sclerosis.

Methods

Patients

102 patients with neurological disease were included in the study. In response to an article in the Journal of the Dutch Society of Multiple Sclerosis, 65 multiple sclerosis patients volunteered to undergo lumbar puncture. Fifty-one patients in whom a diagnosis of clinically definite multiple sclerosis could be made were included in the study. Multiple sclerosis patients were classified as having relapsing remitting (RR), secondary progressive (SP) or primary progressive (PP) disease according to previously published criteria (Lublin and Reingold, 1996).

The control group consisted of 51 patients with the following conditions: one patient had aphasia, one ataxia, one back pain, one benign intracranial hypertension, one chorea, two cerebral infarction, two dementia, one dysphagia, twelve headache, four motor symptoms, two peripheral neuropathies, one sarcoid, one transient ischaemic attack, and twenty-one non-specific sensory symptoms presumably with a functional basis. These samples were obtained from a CSF library from patients undergoing diagnostic lumbar punctures at the National Hospital for Neurology and Neurosurgery, London. The CSF samples were coded and made anonymous in accordance with the MRC (Medical Research Council) guidelines on the ethical use of biological specimen collections in clinical research.

Patient demographics and baseline characteristics are shown in **Table 1**.

Clinical assessment

The Amsterdam group assessed all the multiple sclerosis patients. An ambulation index (AI) (Amato and Ponziani, 1999), an Expanded Disability Status Scale score (EDSS) (Kurtzke, 1983) and a 9-hole PEG test (9HPT) for both hands (Amato and Ponziani, 1999; Kalkers et al., 2000) were performed on all patients within 1 week of the lumbar puncture. The AI classified the gait on a scale ranging from 0 (no impairment) to 9 (restricted to wheelchair without independent transfer). The 9HPT is a measure of upper limb motor function. The 9HPT was performed twice with each hand. The quickest performance for each hand was taken to calculate an average value (Kalkers et al., 2000). Samples of CSF were obtained by routine lumbar puncture. Aliquots of CSF were stored at –70°C until assayed. Approval for the study was

obtained from the Ethics Committee of the VU Medical Centre and The Joint Medical Ethics Committee of The Institute of Neurology and The National Hospital for Neurology and Neurosurgery. Written informed consent was obtained from all multiple sclerosis patients.

Table 1.

Demographic and clinical data [median (range), number]

	Control	MS	Clinical classification		
			RR	SP	PP
Age(years)	41 (27-63), 51	46 (27-65), 51	40 (27-55), 20	46 (28-65), 21	51 (43-55), 10
Gender (M:F)	37/14	28/23	11/9	10/11	7/3
Ambulation Index	NA	4.5 (0-10), 44	1.5 (0-10), 18	6.5 (1-9), 18	4 (1-9), 8
EDSS	NA	3.5 (0-8), 51	2 (0-6.5), 20 [a, b]	6 (1-8), 21 [b]	6 (2-8), 10 [a]
9-HPT	NA	25 (17-84), 49	20 (18-29), 18 [c]	29 (17-84), 21 [c]	26 (17-36), 10

Multiple sclerosis patients are classified into clinical subtypes, NA=not applicable, F=female M=male;

b) difference between RR and PP MS, $p<0.001$
c) difference between RR and SP MS, $p<0.001$
d) difference between SP MS and PP MS, $p<0.05$

Brain tissue preparation

Material

Post-mortem unfixed brain tissue was obtained from 12 clinically and histologically definite multiple sclerosis patients and eight controls. The Multiple Sclerosis Society Tissue Bank at the Institute of Neurology kindly provided these specimens. All multiple sclerosis cases were classified as SP with significant disability (Gveric et al., 2001). The mean age (and range) of the multiple sclerosis patients was 48.6 (29–65) years, with mean disease duration of 19.5 (7–43) years and post-mortem interval of 30.2 (9–52) h. The mean age in the control group was 56.7 (37–71) years and the mean post-mortem interval 26.9 (1–40) h. The brain tissue from multiple sclerosis patients was histologically classified into normal-appearing white matter (NAWM), acute lesions (AL), subacute lesions (SAL), chronic lesions (CL) and grey matter

(GM) using previously published criteria (Li et al., 1993). Control grey and white matter (WM) was obtained from normal subjects without neurological diseases. Adjacent pieces of each type of tissue were excised and homogenized for BSP analysis.

Immunohistochemistry

For immunohistochemistry, sections were immunoperoxidase stained with antibodies directed against GFAP (Newcombe et al., 1986), 14E for oligodendrocytes and reactive astrocytes (Newcombe et al., 1992). Cryostat sections were fixed in methanol (–20°C,
10 min), incubated with primary antibody overnight (4°C) and stained using a three-step peroxidase method.

Protein extraction

Snap-frozen blocks of brain and spinal cord from multiple sclerosis and control cases (0.5–1 g wet weight) were finely cut and resuspended at 1: 5 g/ml in Tris–HCl buffer (100 mM Tris pH 8.1 with 1% Triton X-100). Samples were homogenized on ice by sonication, triturated three times through 19 and 21 gauge needles, and spun at 20 000 *g*. The supernatant was stored at –70°C. Total protein concentration was determined using the Lowry method.

Assays

Brain-specific proteins

S100B (Green et al., 1997 b), ferritin (Keir et al., 1993) and GFAP (A.Petzold et al., unpublished) were measured using in-house ELISA techniques. Albumin in CSF and serum concentrations was determined by a standard Laurell ‘rocket’ electro-immunoassay.

GFAP

Ninety-six-well microtitre plates were coated with SMI26 (Sternberg Monoclonals) in 0.05 M carbonate buffer. The plates were washed with 0.67 M barbitone buffer containing 5 mM EDTA, 0.1% BSA (bovine serum albumin) and 0.05% Tween. The plates were blocked with 1% BSA and washed. CSF was diluted in 0.67 M barbitone buffer

containing {3- [(3-cholaminodopropyl) dimethylammonio]-1-propanesulfonate, CHAPS} and EDTA. The plate was incubated with a horseradish peroxidase (HRP)-conjugated cow polyclonal anti-GFAP (Dako, Denmark) diluted in barbitone buffer containing 5 mM EDTA. After washing, the TMB colour reaction was stopped with 1 M hydrochloric acid. Absorbance was read at 450 and 600 nm. All samples were processed in duplicate. The antigen concentration was calculated from an internal standard curve ranging from 0 to 100 pg/ml. The inter-assay coefficient of variation was <10%.

Oligoclonal bands

CSF and serum oligoclonal immunoglobulin G (IgG) bands were detected using isoelectric focusing (Keir et al., 1990; Andersson et al., 1994).

Statistical analysis

All statistical analyses and graphs were done using SAS software (SAS Institute, Inc., Cary, NC, USA). All mean values are given ±SD or SEM as appropriate. The box (median and 25–75% cumulative frequency) and whisker (1–100% cumulative frequency) are shown in the graphs. The linear relationship between continuous variables was evaluated using the Spearman correlation coefficient (α= 0.05). Linear regression analysis was performed using the least-squares method. Independent variables were compared using the non-parametric two-sample exact Wilcox rank-sum test or the unbalanced two-way ANOVA (general linear model) for more than two groups (Cody and Smith, 1997). Trend analysis was done using the Mantel–Haenzel (M–H χ^2) test (Cody and Smith, 1997). For small sample sizes, levels of significance revealed by either non-parametric method were checked on a categorical level by the Fisher's exact test
(α = 0.05). The cut-off for categorical data analysis was set to the 100% cumulative frequency of the indicated control group. *P* values of <0.05 were considered significant.

Results

CSF study

Oligoclonal bands

The CSF and serum isoelectric focusing patterns were classified according to whether the patients had evidence of intrathecal IgG synthesis (OCB+), evidence of a systemic oligoclonal response with matched bands in the serum and CSF (OCB*), i.e. a 'mirror pattern', or no evidence of an intrathecal or systemic oligoclonal 'IgG' response (OCB–). Forty-six out of 51 multiple sclerosis patients (90%) were classified as OCB+, four (8%) were OCB* and one (2%) was OCB–. All of the control patients were OCB–.

Brain-specific proteins

CSF levels of BSP did not correlate with age, age at onset of disease, disease duration or time from last relapse in the multiple sclerosis patients. In the subgroup analysis, CSF ferritin levels in SP multiple sclerosis patients correlated with disease duration ($r = 0.46$, $p < 0.05$). The CSF to serum albumin ratio was normal.

Clinical subtypes

Relapsing disease

RR multiple sclerosis patients had higher CSF S100B and GFAP levels compared with the other clinical subtypes. S100B was significantly different between the clinical subtypes and controls [$F(3,105) = 2.77$, $p< 0.05$; **Table 2**]. The post-hoc analysis showed that this significance originated from higher levels present in RR multiple sclerosis patients when compared with control patients ($p < 0.01$). Mean S100B levels in RR patients were nearly

2-fold higher compared with PP and 1.5-fold higher compared with SP multiple sclerosis patients, but these differences did not reach statistical significance (data not shown). There was, however, a trend for a stepwise increase of S100B levels from PP to SP to RR multiple sclerosis patients. None (0 out of 10) of the PP, 14% (3 out of 21) of the SP and 35% (7 out of 20) of the RR multiple sclerosis patients had S100B levels above the cut-off of 0.39 ng/ml. This trend for linear increase was significant (M–H $\chi^2 = 5.633$, $P < 0.05$). Importantly, S100B did not correlate with time from last relapse in either clinical subtype.

Table 2.

CSF levels of S100B, ferritin and GFAP in RR, SP and PP multiple sclerosis patients [median (range, number)]

	Control	MS	Clinical classification		
			RR	SP	PP
S100 b (ng/ml)	0.25 (0-0.4), 51 [a,b]	0.3 (0.1-2), 51 [a]	0.3 (0.1-2), 20 [b]	0.27 (0.2-1.4), 20	0.25 (0.1-0.4), 10
Ferritin (ng/ml)	5 (3-7), 51	5 (1-20), 51	4.5 (1-20), 20	6 (1-19), 21	5.5 (3-13), 10
GFAP (pg/ml)	1 (0-10), 51 [c]	3 (10-16), 51	3 (0-11), 20	2 (0-16), 21 [c]	0.5 (0-11), 10

S100B significantly distuinguishes between multiple sclerosis and control patients (F (3,98)=3.09, p<0.05), with RR multiple sclerosis patients being the main contributor (post-hoc analysis). SP multiple sclerosis have significantly higher levels than control patients
(post-hoc analysis only; F (3,98)=2.27, not significant);

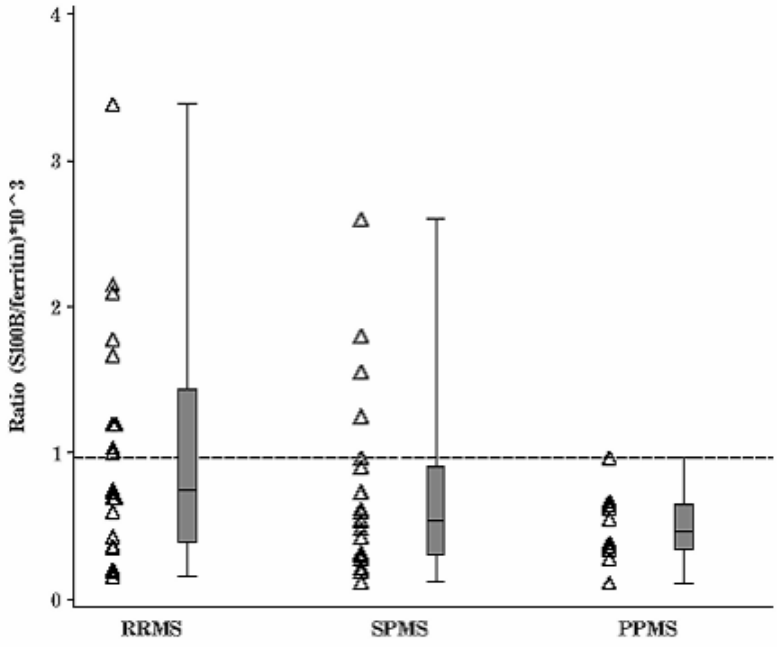
a) difference between control and MS, p<0.05;
b) difference between control and RR MS, p<0.01;
c) difference between control and SP MS, p<0.01

Progressive disease

SP patients had the highest CSF ferritin levels of the clinical subtypes. Ferritin levels in progressive patients (SP and PP) were higher than in RR multiple sclerosis patients, which is the inverse of the levels observed for S100B. Consequently a ratio of S100B: ferritin was able to distinguish significantly between clinical subtypes [$F(3,98) = 6.45$, $p < 0.001$]. The S100B: ferritin ratio was significantly higher in RR (1.0 ± 0.8) than in SP (0.7 ± 0.6), PP (0.5 ± 0.3) or control (0.5 ± 0.2) patients ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; **Figure 1**). None (0 out of 10) of the PP, 19% (4 out of 21) of the SP and 45% (9 out of 20) of the RR multiple sclerosis patients had a S100B: ferritin ratio above the cut-off. The trend analysis revealed a significant linear increase of the S100B: ferritin ratio from PP to SP to RR multiple sclerosis patients (M–H χ^2 = 7.7, $p < 0.01$). In PP patients, ferritin and GFAP were slightly elevated but the difference from the control patients did not reach statistical significance. S100B in PP multiple sclerosis patients were similar to the control group.

Figure 1.

Scatter and box-whisker plot for the CSF (S100B : ferritin) x 10–3 ratio, which was significantly higher in RR than in SP ($P < 0.05$) or PP ($P < 0.01$) multiple sclerosis patients. Zero % of PP, 19% of SP and 45% of RR multiple sclerosis patients had S100B levels above the cut-off of 0.97 (dotted line). The linear stepwise increase is significant ($P < 0.01$)



Controls

CSF S100B was significantly higher in multiple sclerosis patients compared with control patients ($P < 0.05$; Table 2). CSF GFAP levels did not distinguish significantly between control and multiple sclerosis patients. CSF ferritin levels were generally higher in multiple sclerosis patients compared with control patients, but this was not significant.

Disability

Patients were categorized according to the frequency distribution on the clinical scales. The distribution of the AI and EDSS was trimodal. Patients were therefore classified accordingly into those with good (AI ≤ 2), moderate (3–6) and poor (≥7) ambulation. The EDSS was classified into patients with mild (0–3), moderate (3.5–6.5) and severe disability (7–10).

AI

There was a significant difference in CSF GFAP levels between multiple sclerosis patients classified according to the AI and control patients [$F(3,64) = 5.49$, $p < 0.001$; **Table 3**]. In the post-hoc analysis, multiple sclerosis patients with poor ambulation had significantly higher CSF GFAP levels than control patients ($p < 0.001$) or

multiple sclerosis patients with good ambulation ($p < 0.05$). The subgroup analysis revealed that this significance was due to the nine SP multiple sclerosis patients with poor ambulation; these patients had nearly 6-fold elevated median GFAP levels when compared with control patients
($p < 0.05$; **Table 3**). Significantly elevated GFAP levels were also present in poorly ambulating RR multiple sclerosis patients compared with control patients ($p < 0.01$), but not in poorly ambulating PP multiple sclerosis patients.

In SP multiple sclerosis patients, disability measured by the AI correlated with levels of GFAP ($r = 0.57$, $p < 0.01$; **Figure 2**). The 100% cumulative frequency (2 pg/ml) of the CSF GFAP levels of patients with good ambulation was taken as cut-off for the trend analysis. No (0 out of 4) patients with good ambulation, 40% (2 out of 5) of patients with moderate ambulation and 78% (7 out of 9) of patients with poor ambulation had CSF GFAP levels above this cut-off. The trend analysis revealed a significant linear increase within these three AI categories (M–H $\chi^2 = 6.6$, $p < 0.01$).

EDSS

There was a significant difference in CSF GFAP levels between multiple sclerosis patients classified according to the EDSS and control patients [$F(2,57) = 5.06$, $p < 0.01$; **Table 3**]. Severely disabled multiple sclerosis patients had significantly higher GFAP levels than control patients ($p < 0.01$). The post-hoc analysis revealed that this was caused by the approximate 6-fold elevation in median GFAP levels in severely disabled SP multiple sclerosis patients when compared with control patients ($p < 0.01$). The post-hoc analysis also revealed significantly elevated GFAP levels in severely disabled multiple sclerosis patients when compared with moderately disabled patients ($p = 0.05$). There was no linear correlation between the EDSS and GFAP levels and the trend analysis was negative.

In SP multiple sclerosis patients, ferritin correlated with the EDSS ($r = 0.45$, $p < 0.05$). Because of the previously indicated correlation between disease duration and ferritin levels, a partial correlation correcting for disease duration was performed that abolished the correlation between EDSS and ferritin.

9HPT

CSF ferritin was ~2-fold higher in patients with a test performance >55 s (12.3 ± 5.1 ng/ml) than in 'quick' (6.2 ± 4.8 ng/ml) patients ($p < 0.05$, Wilcox rank sum test). Because there are only four 'slow' patients the results were checked by the Fisher's exact test, and no significance could be demonstrated. CSF S100B correlated negatively with the 9HPT in PP multiple sclerosis patients ($r = -0.85$, $p < 0.01$; **Figure 2**).

Brain tissue study

Grey matter

GFAP and S100B were elevated 2–3-fold in multiple sclerosis GM compared with control GM (**Figure 3; Table 4**). Significantly more multiple sclerosis GM samples had S100B and GFAP levels above the cut-off when compared with control GM ($p < 0.001$ and $p < 0.05$, respectively).

Ferritin levels were higher in multiple sclerosis GM than in control GM. There were, however, no differences between multiple sclerosis GM and multiple sclerosis WM, or control GM and control WM ferritin levels.

Table 3.

CSF GFAP levels (pg/ml) in control, multiple sclerosis patients and clinical subtypes [median (range), number]

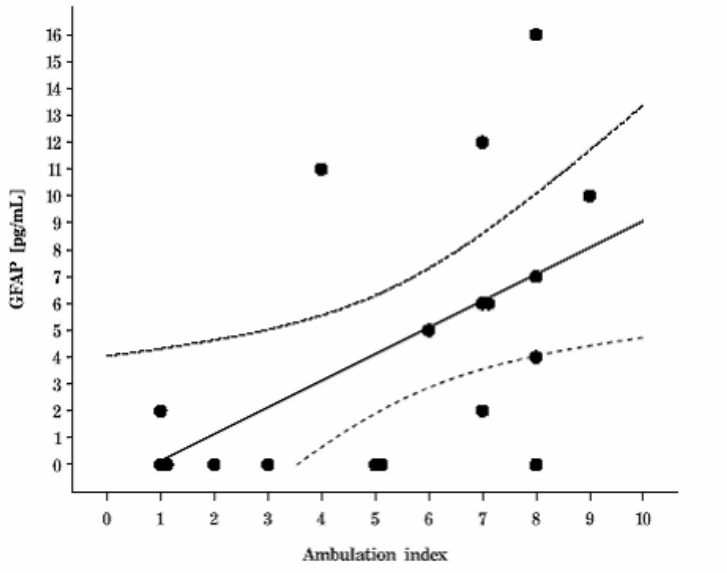
Clinical subtype	GFAP (pg/ml)	Ambulation Index		
		<2	2-6	>6
Control	1 (0-13), 51			
MS		2 (0-11), 19	0 (0-11), 8	6 (0-16), 17 [a, b]
SP		0 (0-2), 4	0 (0-22), 5	6 (0-16), 9 [a, b]
PP		0 (0-11), 3	4.5 (0-9), 2	1 (0-10), 3
RR		3 (0-10), 12	0 (0), 1	6 (3-11), 5 [c]
		EDSS		
		<3.5	3.5-6.5	>7
MS		3 (0-11), 27	0 (0-11), 15	6 (0-16), 9 [d,e]
SP		2 (0-10), 5	0 (0-11), 9	6 (0-16), 7 [d]
PP		0 (0-11), 4	4.5 (0-10), 4	5.5 (1-10), 2
RR		3.5 (0-11), 18	0 (0), 2	NA

GFAP significantly distuinguish grades of disability in clinical subtypes (post-hoc analysis) from control patients (AI: $F(3,64)=5.49$, $p<0.001$; EDSS: $F(2,57)=5.06$, $p<0.01$)

a) difference between control and AI>6, $p<0.001$
b) difference between AI<2 and AI>6, $p<0.05$
c) difference between control and AI>6, $p<0.01$; d difference between control and EDSS>7, $p<0.01$;
d) difference between EDSS between 3.5-6.5 and EDSS>7, $p<0.05$

Figure 2.

Disability.
(A) CSF GFAP levels correlated significantly with the AI in SP multiple sclerosis patients ($r = 0.57$, $P < 0.01$). Data points are placed adjacent to each other if observations overlapped.
(B) CSF S100B levels correlated significantly with the 9HPT of the dominant hand in PP multiple sclerosis patients ($r = –0.85$, $P < 0.01$). The linear regression line, and the 5% lower and 95% upper confidence curves are shown.



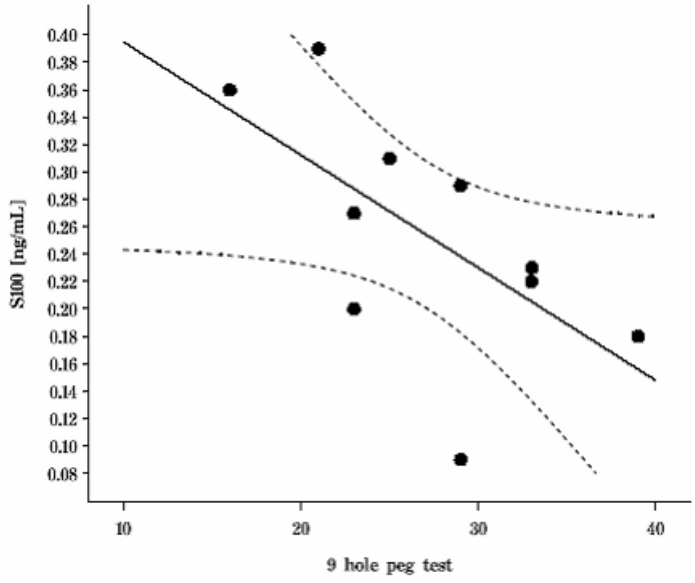
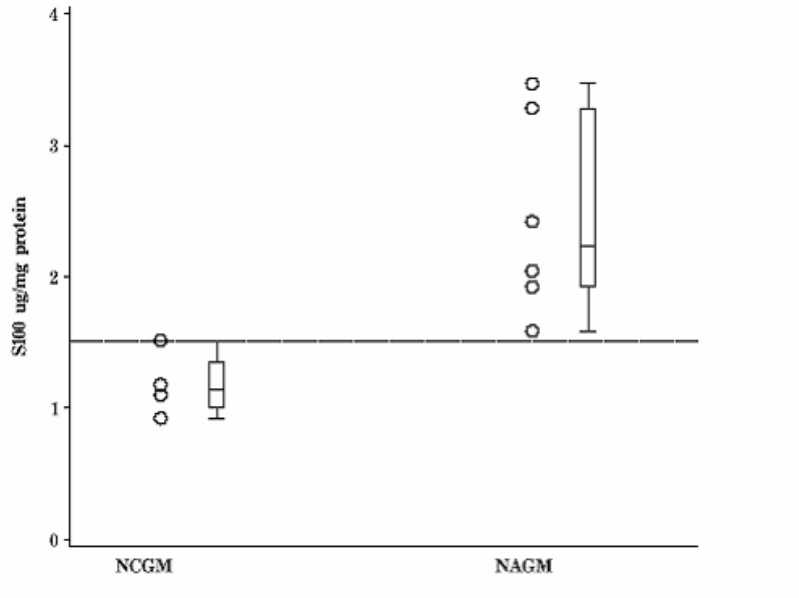


Figure 3.

(A) When comparing levels of GM S100B in cortical multiple sclerosis patients (NAGM) with controls (NCGM), a 2-fold increase is observed. Significantly more cortical samples from multiple sclerosis patients than controls had S100B levels above the cut-off
(dotted line; $P < 0.001$).



(**B**) When comparing levels of GM GFAP in cortical multiple sclerosis patients (NAGM) with controls (NCGM), a 3-fold increase is observed. Significantly more cortical samples from multiple sclerosis patients than controls had GFAP levels above cut-off (dotted line; $P < 0.05$).

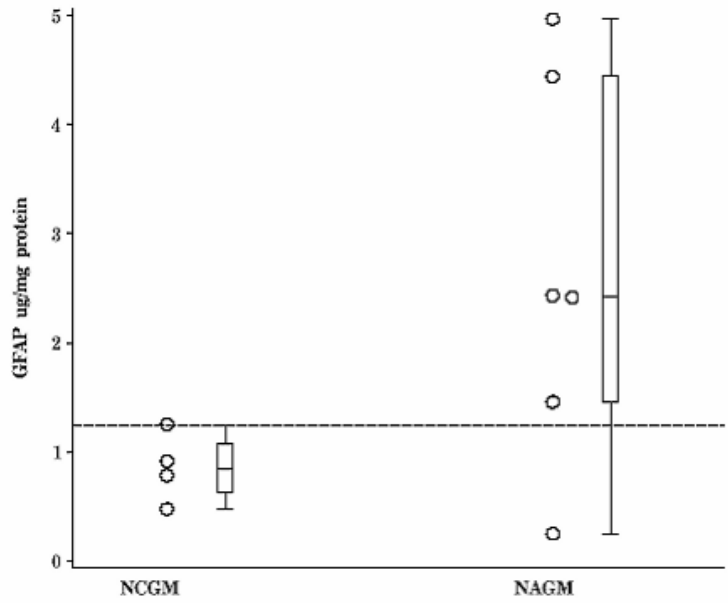

GFAP ug/mg protein
5
4
3
2
1
0
NCGM
NAGM

Table 4.

Levels of S100B, GFAP and ferritin (in µg/mg protein) in homogenized brain tissue

Protein (µg/ml)	White matter				
	Control	NAWM	AL	SAL	CL
S100B	2.4 (1.5-5.6), 5	4.8 (2.6-7.1), 5	5.2 (4.0-6.4), 6 [a]	3.4 (2.4-4.1), 4 [a]	3.9 (2.0-8.0), 7
GFAP	1.7 (1.1-5.9), 5	1.4 (0-6.0), 5	5.3 (2.6-6.7), 6	3.9 (2.5-5.4), 4	4.0 (0.4-11.4), 7
Ferritin	3.6 (2.5-4.7), 5 [b]	7.0 (4.8-9.4), 5 [b]	5.2 (3.7-11.2), 6	5.1 (3.9-7.0), 4	5.7 (4.0-13.2), 7
Protein (µg/ml)	Grey matter				
	Control		Multiple Sclerosis		
S100B	1.1 (0.9-1.5), 4 [c]		2.2 (1.6-3.5), 6 [c]		
GFAP	0.8 (0.5-1.2), 4 [d]		2.4 (0.2-5.0), 6 [d]		
Ferritin	3.1 (1.3-4.1), 4		4.8 (2.5-12.3), 6		

The median (range, number) values for WM control tissue, NAWM, AL, SAL and CL, and values for GM from control and multiple sclerosis tissue are shown;

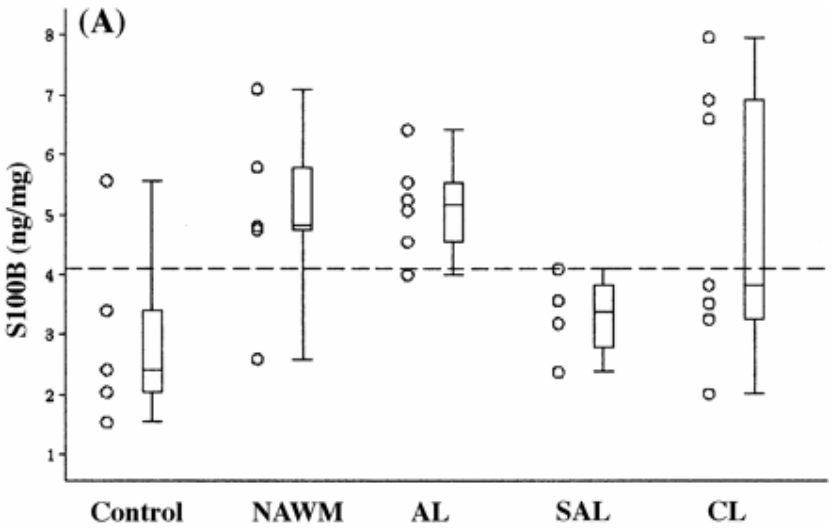
a) difference between AL and SAL, $p<0.001$;
b) difference between control and NAWM, $p<0.05$;
c) difference between control and MS, $p<0.001$;
d) difference between control and MS, $p<0.05$

White matter

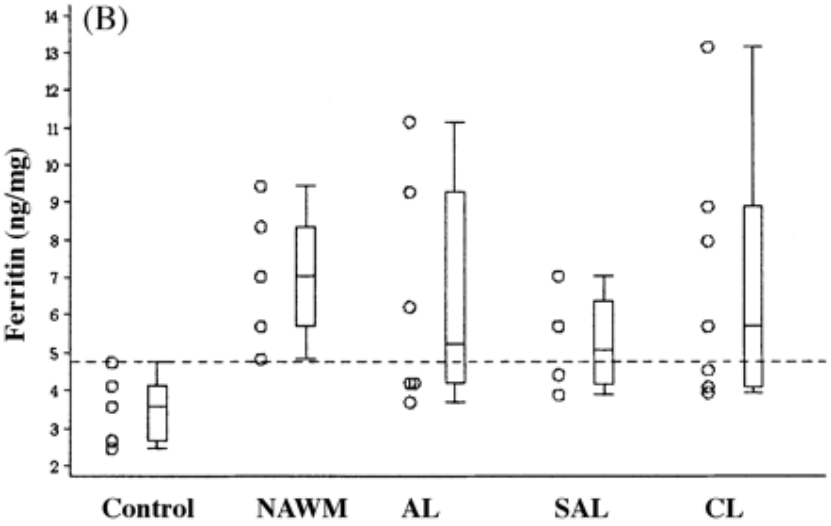
S100B levels were ~2-fold higher in acute plaques than in SAL (**Figure 4A**). Significantly more AL than SAL lesions had S100B levels above the cut-off ($p < 0.001$). Ferritin levels were higher in all multiple sclerosis lesion types compared with controls (**Figure 4B**). A significantly higher proportion of samples from NAWM had ferritin levels above cut-off when compared with control WM tissue ($p< 0.05$).

Figure 4.

(A) White matter S100B levels. Significantly more samples from acute plaques than from subacute plaques have S100B levels above the cut-off (dotted line; $P < 0.001$)



(B) White matter ferritin levels. Significantly more samples from NAWM had ferritin levels above the cut-off (dotted line) compared with control white matter ($P < 0.001$)



Discussion

Clinical subtypes

Our findings show that biomarkers for astrocytic (CSF S100B) and microglial (CSF ferritin) activation have the potential to distinguish between patients with RR and progressive (PP and SP) multiple sclerosis. There was a significant trend for increasing S100B levels from PP to SP to RR multiple sclerosis patients, with RR multiple sclerosis patients having significantly higher S100B levels than control patients. Ferritin levels were significantly higher in SP patients than in control patients (**Table 2**). Consequently, a S100B/ferritin ratio was capable of distinguishing RR from SP multiple sclerosis patients. This suggests that astrocytic and microglial activation predominate in relapsing and progressive disease, respectively (**Figure 1**). Multiple sclerosis patients had significantly elevated CSF S100B levels compared with control patients, which was principally due to the high S100B levels in RR multiple sclerosis patients. This confirms the results of most previous studies (Michetti et al., 1980; Massaro et al., 1985, 1997; Lamers et al., 1995). The median relapse-free interval in our study was 15 months in RR and 77 months in SP multiple sclerosis patients. Therefore, CSF S100B levels represent relapse-independent astrocytic activity. *In vitro* astrocytes have been shown to increase their expression of S100B after exposure to adrenocorticotropic hormone (Suzuki et al., 1987), which has proved to be an effective drug in the treatment of multiple sclerosis (Filippini et al., 2000). In line with these findings would be the high S100B levels in NAWM, which further supports the concept of relapse-independent disease activity. The dynamics of

S100B clearance from the extracellular space via the CSF are, however, unknown, but one would assume that due to dilution the extracellular concentration at the site of release would be much higher than that in the CSF. Nanomolar levels of S100B have neurotrophic properties, while micromolar S100B levels have cytotoxic properties (Donato, 2001). Nave's group described how glial pathology precedes axonal degeneration, but the mechanism of progress from one to the other is not fully understood (Griffiths et al., 1998). MRI techniques (Brex et al., 1999; Tourbah et al., 1999) and immunohistochemistry (Trapp et al., 1998; Bjartmar et al., 2001) provide compelling evidence of disease activity in NAWM. The pathological features underlying changes in NAWM need to be clarified. In a post-mortem study combining MRI and histology studies, de Groot et al. (2001) suggest that such changes could indicate '(p) reactive' lesions. Filippi (1998) recently presented evidence that quantitative changes in magnetization transfer can be observed weeks before the development of enhancing lesions.

It is of note that elevated levels of S100B due to head injury or rapid parenchymal destruction have been reported in patients with epilepsy (Steinhoff et al., 1999), Creutzfeldt–Jakob disease (Otto et al., 1997), stroke (Aurell et al., 1991; Wunderlich et al., 1999) and acute brain injury (Herrmann et al., 2000). These levels should, however, be distinguished from those in slowly progressive diseases where S100B might play a different pathogenic role, i.e. by modulating the inflammatory response through stimulation of inducible nitric oxide synthase (Adami et al., 2001; Donato, 2001) or modify disease progression by yet unknown mechanisms, e.g. in Down's syndrome or Alzheimer's disease (Griffin et al., 1989; Green et al., 1997a; Donato, 2001). Although extracranial sources of S100B, such as adipose tissue, testis and skin, are known (Hidaka et al., 1983; Takahashi et al., 1984; Michetti et al., 1985) and are potential confounding factors in the interpretation of serum S100B levels (Donato 2001; Jackson et al., 2001), this is not likely to affect reported levels of CSF in this study because the blood–brain barrier (as determined by CSF/serum albumin ratio) was intact and all multiple sclerosis patients were beyond acute relapse.
In our study, CSF ferritin is significantly higher in SP multiple sclerosis patients than in the control group. To our knowledge only one other study has measured ferritin levels in multiple sclerosis patients and found them to be elevated in progressive patients (LeVine et al., 1999). This result is supported by the results of our brain tissue

study. Ferritin concentrations were higher in all lesion types of progressive multiple sclerosis brains when compared with control WM. This was significant for NAWM versus control WM (**Figure 4B**). As NAWM contributes the bulk of brain tissue equilibrating with the CSF, this result is not surprising. Interestingly, Hulet et al. (1999) found decreased ferritin binding to white matter within a multiple sclerosis lesion. The oligodendrocyte requires iron for the synthesis of myelin (Connor and Menzies, 1996), therefore upregulated ferritin levels in multiple sclerosis brain could reflect a physiological reaction to decreased binding and metabolic needs.

In contrast to one study using the CSF of five healthy volunteers as controls (Rosengren et al., 1995), we and others (Albrechtsen et al., 1985; Noppe et al., 1986) found no overall significant difference between CSF GFAP levels in multiple sclerosis patients and a control group, consistent with patients with other neurological disorders. However, significantly elevated GFAP levels compared with our controls were found in multiple sclerosis patients with poor ambulation (AI ≥7) or severe disability (EDSS >6.5). GFAP has also been found to be elevated in dementia (Eng and Ghirnikar, 1994), normal pressure hydrocephalus (Albrechtsen et al., 1985), asphyxiated newborns (Blennow et al., 1995), post head injury (Missler et al., 1999), brain infarction (Aurell et al., 1991) Lyme-borreliosis (Dotevall et al., 1999), trypanosomiasis (Lejon et al., 1999) and multiple sclerosis (Rosengren et al., 1995). GFAP should therefore be regarded as a non-specific biomarker of CNS tissue injury.

Disability

Patients with poor ambulation had significantly higher CSF GFAP levels than patients with good ambulation and control patients (**Table 3**). Also severely disabled patients had significantly higher CSF GFAP levels compared with mildly disabled patients. This is suggestive of increased astrogliosis within the spinal cord of poorly ambulating or disabled patients. Compared with the control group, only these patients had significantly higher GFAP levels. The subgroup analysis revealed that this was most marked within patients with SP multiple sclerosis. This study revealed a significant correlation between GFAP and individual AI scoring for SP multiple sclerosis patients ($r = 0.57$; **Figure 2**). The lack of correlation in PP multiple sclerosis may relate to the small number of cases studied. We interpret the results as demonstrating a direct relationship between GFAP and astrogliosis, which is expressed clinically as disability.

The reason why a direct correlation was found between GFAP and individual points on the AI but not with the EDSS can be explained by the physiological basis of these clinical scales. The AI essentially measures gait. The EDSS, on the other hand, includes other neurological functions that are outside (rostral) the anatomical parts of the CNS that equilibrate with the CSF in the lumbar sac. This 'CSF analytical brain' consists of the inner half of the telencephalon, the basal cortex, the cerebellum, the brain stem and the spinal cord (Felgenhauer and Beuche, 1999). Each lost axon innervating the lower limb could potentially be replaced by a gliotic scar of 1 m in length (Kreutzberg, 1995), which is the source of GFAP release, and would parallel the decline in ambulation. Thus, almost all changes measured by the AI, but only some assessed by the EDSS, would be reflected in a change in the level of lumbar CSF GFAP.

This was also demonstrated by the study of Rosengren et al. (1995), who studied serial CSF samples in 10 RR multiple sclerosis patients. The scale applied to assessing disability, the RFSS (regional functional score system), includes visual and mental functions. Contradictory changes in the RFSS and lumbar CSF GFAP levels were observed in eight of the 10 patients studied. Importantly, this study of serial CSF samples (seven lumbar punctures per patient over a 2-year period) did not reveal any relationship between the level of CSF GFAP and the time from relapse.

It is difficult to explain the strong negative correlation between S100B and the 9HPT, which has not previously been observed in PP multiple sclerosis patients. The neurotrophic role of S100B in nanomolar concentrations, however, is well described (Haglid et al., 1997; Donato, 2001). One might speculate on whether there is an association between the treatment responses to adenocorticotropic hormone in multiple sclerosis (Fillippini et al., 2000) that upregulates S100B excretion in vitro (Suzuki et al., 1987). Certainly moderately elevated S100B levels in multiple sclerosis could be an indicator for moderate astrocytosis, which might be beneficial. In this context, high levels would have to be associated with relapse and possible toxic effects, while low levels would be related to 'burn out'. These results need to be confirmed in other groups to assess whether the correlation is a consistent finding.

Brain tissue study

The levels of all BSP appear to be increased in multiple sclerosis GM (**Figure 3**). This was significant for S100B and GFAP. This finding is particularly relevant for studies

focusing on the cognitive and neuropsychiatric aspects of multiple sclerosis. The results of studies examining BSP in GM, however, should be interpreted with caution. The cortex does not form part of the 'CSF analytical brain' (Felgenhauer and Beuche, 1999) since cortically derived BSP will flow into the CSF and will be absorbed by the rostral arachnoid villi. It is unlikely that BSP released from the cortex will be detectable in the lumbar CSF. It might, however, be possible to measure changes in cortical BSP in other body fluids (Thompson and Green, 1998).

The significantly higher levels of S100B in AL compared with SAL suggest that S100B expression is predominantly upregulated in the acute phase of the disease and returns to normal in at least half of all patients (**Figure 4A**). In contrast, ferritin levels are consis-tently higher in multiple sclerosis than in control brain tissue and were found to be significantly higher in multiple sclerosis NAWM compared with control WM (**Figure 4B**). This is parallelled by S100B but not by GFAP, supporting the idea that GFAP might be more relevant as a biomarker for damaged tissue. The results of the S100B and ferritin analysis point to early astrocytic activation, which may return to normal despite continuing microglial activation in multiple sclerosis WM (**Figure 4**), as well as the striking elevation in the cortex of S100B and GFAP (**Figure 3**).

Conclusion

The first hypothesis of this cross-sectional study, that there is a relationship between biomarkers for glial reaction and clinical subtypes, was confirmed. In the relapsing phase of the disease, S100B is elevated, while high CSF ferritin levels are observed in all phases. The second hypothesis that BSP levels relate to disability is true for GFAP. GFAP correlates with the AI and EDSS. Finally it was shown that BSP levels relate to histopathological features of CNS lesions, which allows us to draw certain conclusions regarding CSF findings. The question of whether these biomarkers prove useful as outcome measures in future treatment trials needs to be addressed in further prospective studies.

Chapter 3.2

Multiple sclerosis: neurofilament light chain antibodies are correlated to cerebral atrophy

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Abstract

Markers of axonal damage in cerebrospinal fluid (CSF) and serum of patients with different subtypes of multiple sclerosis (MS) were related to measures of disease progression on magnetic resonance imaging (MRI).
In 51 patients with MS (21 relapsing-remitting; 20 secondary progressive; 10 primary progressive), levels of heavy and light neurofilaments (NfH and NfL) and antibodies to neurofilaments (anti-NfL and -NfH), as well as the total immunoglobulin G (IgG) were analyzed. MRI analysis included T2 hyperintense, T1 hypointense and gadolinium enhancing lesions and markers of cerebral atrophy (ventricular and parenchymal fractions). For the total group, correlations were found between the anti-NfL index and the parenchymal fraction (PF: $r= -0.51$, $P<0.001$), T2 lesion load (T2LL: $r=0.41$, $p<0.05$), ventricular fraction (VF: $r= 0.37$, $p<0.05$) and the T1 Lesion Load (T1LL: $r= 0.37$, $p<0.05$). For the anti-NfH index, a correlation was found with the PF ($r= -0.39$, $p<0.05$). No correlations were found between the IgG index and MRI measures. This study suggest that intrathecal production of anti-NfL antibodies may serve as a relevant marker of tissue damage, particularly axonal loss in MS.

Introduction

Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS), but recent evidence suggests that the role of axonal degeneration has long been underestimated ((Martin et al., 1995). The disease has a highly variable course both within and between patients (Ebers et al., 2000), suggesting that there might be a considerable amount of disease heterogeneity. Heterogeneity in lesions has been shown in both magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS) studies (Brex et al., 2000: Lee et al., 1999), as well as in pathologic studies, suggesting different patterns of demyelination and axonal damage (Lucchinetti et al., 2000). Axonal damage seems to play a key role especially in relation to chronic irreversible neurological disability. Pathologically it can be demonstrated not only in chronic lesions but also in early lesions (Trapp et al., 1998); on MRI it can be detected as atrophy and hypointense T1 lesions. These so-called "black holes" have been shown to reflect a more destructive type of lesion in which axonal damage is an important feature, whereas T2 lesions seem to reveal a much wider range of pathology (van Walderveen et al., 1998). Neurological disability in patients with MS has been correlated with atrophy of the spinal cord, cerebellum and cerebrum (Losseff et al.1996a and 1996b, Davie et al., 1995).

Although MRI has the potential to identify atrophy and different types of lesions, it lacks pathologic specificity. Cerebrospinal fluid analysis, however, may add greater pathological specificity as it provides information from the body fluid that is most closely associated to the disease process. It has been suggested that intrathecal levels of axonal cytoskeletal proteins may be reliable markers of axonal damage.

Neurofilaments (Nf) are important axonal cytoskeletal proteins and are intimately linked to axonal diameter and myelination (Fuchs et al., 1998). The 68-kDa neurofilament light subunit (NfL) forms the core of the Nf with the highly phosphorylated 150-kDa medium (NfM) and 200-kDa heavy (NfH) units more peripherally situated. Previous reports have suggested that CSF levels of NfL may be a marker of axonal damage (Semra et al., 2002; Lycke et al., 1998; Trapp et al., 1998) in MS and other neurological diseases (Tullberg et al., 1998). Recently, elevated intrathecal production of antibodies to NfL has been reported in secondary progressive (SP) and primary progressive (PP) MS (Silber et al., 2002).

The purpose of this study was to determine levels of NfL and NfH and their respective antibodies in matched CSF and serum samples from patients with different clinical subtypes of MS and to evaluate their relation to MRI measures of tissue damage.

Materials and Methods

Subjects

Subjects were recruited in response to an appeal in the periodical of the Dutch MS Society. A total of 65 individuals volunteered to undergo both MRI and lumbar puncture (LP). Seven patients had to be excluded, because a definite diagnosis of MS could not be confirmed and seven later refused to undergo a LP, leaving a total of 51 subjects. Subjects were classified as having relapsing remitting (RR), SP or PP disease based on their clinical course (Lublin and Reingold, 1996) at the time of the neurological exam. Disability was measured using the expanded disability status scale (EDSS) (Kurtzke, 1983).

The group consisted of 28 women and 23 men with a mean age at the time of the test of 44.5 years with an interquartile range (IQR) of 38.1 to 51.2. The median disease duration, calculated as the time lapse between the onset of neurological symptoms and the time of testing, was 13.8 years (IQR 7.0-23). The median time from the last relapse until the lumbar puncture was 36.5 months IQR 6.3-81.3 (RR and SP patients only). Samples of CSF were obtained by LP and aliquots of CSF were stored at -80°C. Detailed neurological examination and MRI scanning was performed within one week of the LP. Approval for the study was obtained from the Ethics Committee of the VU Medical Center and written informed consent was obtained from all patients.

Albumin

CSF and serum albumin concentrations were determined by a standard Laurell "rocket" electroimmunoassay. Neurofilament-H and –L an enzyme-linked immunoassay measured measured NfH, by using 96-well microtiter plates (Maxisorb, Nunc, Denmark). The plates were coated with monoclonal anti-NfH antibodies (SMI 35, Sternberger Monoclonals Inc., Lutherville, USA) in 0.05 M carbonate buffer and incubated overnight. The plates were washed with barbitone buffer containing EDTA,

bovine serum albumin (BSA) and polyoxyethylenesorbitan monolaurate (Tween 20, Sigma, Poole, UK). After blocking and washing the plates were incubated with diluted CSF for 1 hour and washed. Then the second antibody was added (rabbit polyclonal anti-NfH, Sigma) and incubated for 1 hour at room temperature and washed. Finally horseradish peroxidase-conjugated polyclonal swine antirabbit immunoglobulin (IgG) antibody was added for 1 hour and washed. TMB (Dako, Carpinteria, CA, one-step substrate) was used as color substrate. The color reaction was stopped with hydrochloric acid and the absorbance read at 450 nm referenced against 750 nm. All samples were processed in duplicate. The antigen concentration was calculated from a standard curve
(range 0-5 ng/mL) using bovine NfH (Affiniti Research Products, Exter, UK). High and low quality controls were run with each plate. The coefficient of variation for 2 ng/mL was 5.6 % and for 0.2 ng/mL 19.8 %.
The same method as for NfH was used to determine NfL with the first antibody being a monoclonal anti-Nf 68 (Sigma). The second antibody used was the rabbit polyclonal anti NfL (Affiniti Research Products).

Anti- NfL & anti- NfH

A sensitive, capture ELISA was used to measure anti-NfL and anti-NfH IgG antibodies in CSF and serum (Silber at al, 2002). In brief, ELISA plates were coated with 50µl of a solution of purified 68-Kd (BL 62008) or 200-Kd (CBL 62010) bovine neurofilament (Cymbus biotechnology, UK; 2.5µg/ml) in bicarbonate coating buffer (pH 9.6) at 4^0C overnight. The plates were blocked with 1% bovine serum albumin in phosphate buffered saline (BSA/PBS) and then coated with CSF or serum samples (50µl/well) for two hours at 37^0C. Samples were analysed in duplicate; CSF undiluted and serum diluted 1/400 in 1% BSA/PBS. Bound antibody was detected with an antibody to human IgG (γ-chain specific) conjugated to alkaline phosphatase (Sigma, A-3150, UK) for 1 hour, washed and then optical densities (ODs) measured after colour development with p-nitrophenyl phosphate.
In developing each assay, we utilized a pool of human sera previously demonstrated to have high titres of anti-Nf and anti-axonal IgG. Serial dilutions of the pooled sera were run in each test to generate a standard curve and to ensure consistency of the assay. For the anti-NfL, the mean between-test coefficient of variation (CV) of the

standard curve was 18.9% and for anti-NfH 17.0%. In the anti-NfL assays, the overall mean CV of all serum and CSF specimens were 7.0% and 8.7%, and in the NfH assays 6.1 and 8.5%. The sensitivity of the assays, determined by the lowest dilution of the standard curve with an OD consistently higher than the mean plus three SD of wells filled with just BSA, was 1/3,200 for the anti-NfL and 1/6,400 for the anti-NfH assays. In order to distinguish intrathecal and systemic production of anti-Nf IgG, an anti-NfL index and anti-NfH index were calculated similar to the calculation of the IgG index (Keir et al., 1993) using the formula: (CSF units/serum units)/ (CSF albumin/serum albumin).

IgG

Total immunoglobulin G was measured in CSF and serum samples by a two-site immun-oenzymetric assay (Cygnus Technologies, Massachusetts, USA). IgG index was calculated as mentioned previously.

MR Imaging and analysis

Brain MRI was performed using a 1.5 T system (Siemens AG, Erlangen, Germany) and consisted of an axial T1 and T2 weighted spin echo MR imaging, with 3 mm slice thickness, and 1 x 1mm in-plane resolution. Brain atrophy and lesion load measurements were analysed on workstation (Sun, Mountainview, California, USA) using semi-automated seed-growing software developed in house, based on local thresholding (Show-Images) (Kalkers et al., 2001).

Two ratios were calculated (i) the parenchymal fraction (PF), defined as whole brain parenchyma/ intracranial volume; and (ii) the ventricular fraction (VF), defined as ventricular volume/ whole brain parenchyma. The total volume of hyperintense lesions seen on the T2 images and of gadolinium-enhancing lesions and hypointense lesions seen on T1-weighted images were calculated. The MRI raters were trained in measuring lesion loads and volumes, with a CV of less than 3%. In two subjects no adequate MRI data could be obtained.

Statistics

Technicians blinded to the results of other analyses performed MRI analysis. The researchers who performed the tests on the samples were blinded for the clinical and MRI data. Data analysis was performed with the SPSS software package (version 9.0

for Windows; SPSS, Chicago, IL). Data were checked to see whether their distribution was normal and non-parametric statistics used when normality was rejected. All correlations were studied using Spearman rank correlation coefficient (r) for non-parametric testing of data. The correlations were analyzed in the MS patients as a whole group, in the separate MS subtypes, as well as in subgroups defined by the presence or absence of relapses in the last 2 years. Differences between groups were compared by one-way analysis of variance, Wilcoxon test, or Kruskall-Wallis test as appropriate. A 5 % level of significance was used throughout.

Results

Clinical and MRI data

The clinical characteristics of the subjects in the different diagnostic groups are shown in **Table 1**. As expected, significant differences were observed between RR and SP with respect to age, disease duration, EDSS and time since last relapse. Significant differences between SP and PP subjects could be shown with respect to the EDSS. The MRI characteristics are summarized in **Table 2**; no significant differences could be shown between the subgroups.

Table 1.

Clinical characteristics of patients expressed as medians (interquartile range)

	All subjects	RR (n= 20)	SP (n= 21)	PP (n= 10)
Male:Female	23:28	9:11	11:10	3:7
Age (yrs)	46.3 (38.2-51.1)	40.0 (32.1-47.7)	46.2 (37.1-51.2)	51.0 (47.8-52.4)
Disease duration (yrs)	13.8 (7.3-20)	7.6 (3.7-12.9)	19.1 (13.3-22.1)	13.4 (9.5-17.8)
EDSS score	3.5 (2.0-6.5)	1.75 (1.0-2.5)	6.0 (4.0-7.0)	6.0 (2.8-6.6)
Time from last relapse (mnths)	36.5 (6.3-81.3)	15.0 (4.3-40)	77 (26-145)	-

RR= relapsing remitting; SP= secondary progressive; PP= primary progressive; n= number of subjects; yrs=years; mnths=months

CSF markers

Results of the CSF assays are shown in Table 3. No significant differences were found between the RR, SP and PP groups for the IgG indices, levels of CSF NfH, or anti-NfL and anti-NfH indices. NfL protein was below the detection limit of the test in all CSF samples. CSF IgG correlated with CSF anti NfL ($r=0.51$, $p<0.01$) and CSF anti NfH ($r=0.41$, $p<0.05$). The IgG index correlated only with the anti- NfH index

(r=0.43, p<0.05), but not with the anti-NfL index. The anti-NfH index correlated highly with the anti-NfL index (r=0.73, p<0.001). There was no corerlation between CSF NfH and either the anti-NfH index or CSF anti-NfH.

Table 2.

MRI characteristics in patients with MS expressed as median (interquartile range)

MRI characteristics	All subjects	RR (n=18)	SP (n= 21)	PP (n= 10)
Brain	0.9	0.6	2.0	0.04
T1 LL (cc)	(0.16-3.3)	(0.1-2.5)	(0.6-3.5)	(0-7.5)
Brain	5.9	3.4	7.6	7.0
T2 LL (cc)	(2.9-11)	(2.4-10.6)	(4.9-17.5)	(0.50-24.3)
Brain	0	0	0	0
Gad LL (cc)	(0-0)	(0-0.12)	(0-0)	(0-0)
Parenchymal	0.80	0.82	0.80	0.80
Fraction	(0.78-0.83)	(0.79-0.84)	(0.78-0.83)	(0.75-0.86)
Ventricular	0.030	0.029	0.035	0.033
Fraction	(0.021-0.043)	(0.020-0.035)	(0.026-0.047)	(0.020-0.051)

RR= relapsing remitting; SP= secondary progressive; PP= primary progressive; n= number of subjects; LL= Lesion Load; Gad= Gadolinium

Table 3.

CSF levels and corresponding indices in patients with MS expressed as median (interquartile range)

	All subjects	RR (n= 20)	SP (n= 21)	PP (n= 10)
NfL (ng/mL)	ND	ND	ND	ND
NfH (ng/mL)	0.05 (0.02-0.15)	0.07 (0.02-0.15)	0.04 (0.02-0.17)	0.09 (0.0.2-0.13)
Anti NfH index	0.29 (0.22-0.37)	0.28 (0.23-0.36)	0.35 (0.23-0.37)	0.23 (0.17-0.37)
Anti NfL index	0.21 (0.16-0.31)	0.20 (0.14-0.27)	0.24 (0.17-0.33)	0.22 (0.17-0.27)
IgG index	0.79 (0.62-1.2)	1.2 (0.61-1.5)	0.78 (0.65-1.2)	0.75 (0.66-1.2)

RR= relapsing remitting; SP= secondary progressive; PP= primary progressive; n= number of subjects; NfL= neurofilament-L protein; NfH= neurofilament-H protein; Anti=antibodies; ND= not detectable

Correlations between CSF and demographics

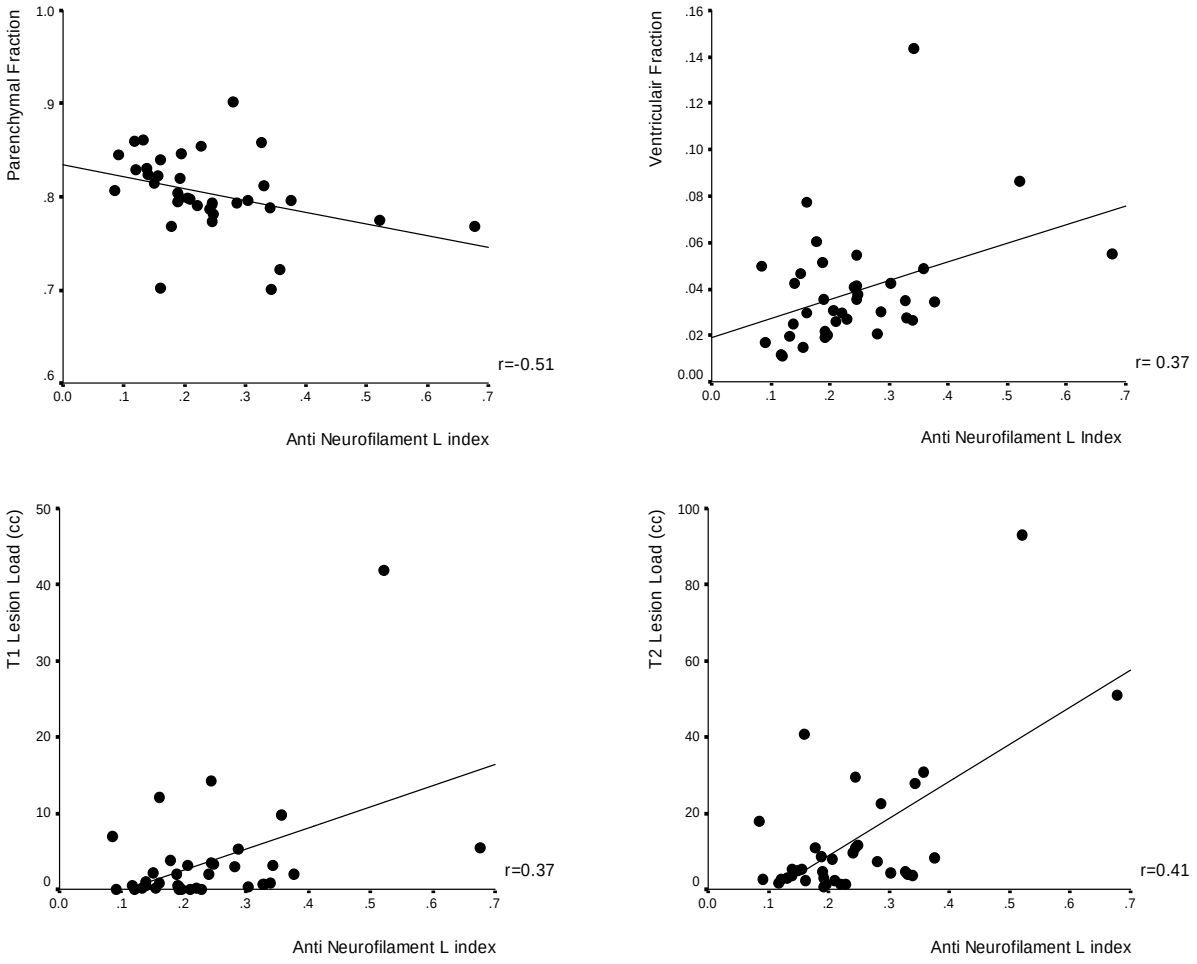
In the group as a whole, there were no significant correlations observed between the CSF Nf, the anti-Nf, or IgG indices and EDSS score, subject age or disease duration.

Correlations between laboratory and MRI measures

In the group as a whole there were correlations between the anti-NfL index and the PF ($r= -0.51$, $p< 0.001$), the T1 lesion load ($r= 0.37$, $p < 0.05$), the T2 lesion load ($r=0.41$, $p<0.05$) and the VF ($r= 0.37$, $p<0.05$) (**Figure 1**). For the anti-NfH index there was only a correlation with the PF ($r=-0.39$, $p<0.05$). There were no correaltions between any MRI measures and either the IgG index or CSF NfH. When testing the subgroups separately we found that correlations were mainly found in the RR subgroup: the anti-NfL index correlated with the PF, VF and T1LL ($r= -0.56$, $p<0.05$; $r= 0.72$, $p<0.01$ and $r=0.61$, $p<0.05$). No significant correlations were found between the CSF proteins or antibodies and MRI measures in the SP and PP groups.

Figure 1.

Correlations between the anti-neurofilament-L index and four MRI measures



Yrs=years; mnths=months; EDSS= expanded disability status scale; RR=relapsing remitting; SP=secondary progressive; PP=primary progressive; n=number of subjects

Discussion

The current study indicates that there is a significant correlation between intrathecal production of IgG antibodies against NfL and MRI markers of inflammation and tissue destruction in MS. The strongest correlation was for the PF, one of the two atrophy measures analyzed in this study. In previous studies levels of neurofilaments or their antibodies have correlated with clinical measures of disease severity, whereas in this study we used MRI. MRI measures are more precise, objective and sensitive to change and may give a more accurate relation to the underlying pathologic process. Measurement of brain atrophy has recently been recognized as one of the most promising methods for documenting the evolution of the disease, especially because it is thought to reflect neurodegeneration, which is more prominent in progressive phases of the disease. Even though measurement of atrophy reflects the end stage of the process that underlies irreversible disability, it is not pathologically specific because both demyelination and axonal loss can inevitably lead to tissue loss. Our current findings provide additional evidence for the relation between atrophy and axonal damage. At least two explanations could be given for our observation that the anti-NfL index is more closely related to atrophy measures rather than to lesion load measures. First, lesion heterogeneity is increasingly recognized as important but not fully reflected in the MRI measures applied in this study and secondly atrophy does not only result from axonal loss inside lesions, but also from secondary degeneration outside lesions, as reflected by decreased levels of N-acetylaspartate on MRS in the so-called normal appearing brain tissue (Tedeschi et al., 2002; van Walderveen et al., 1999; Fu et al., 1996).

Remarkably, when we analyzed correlations between the anti-NfL index and MRI measures in the different subgroups of MS (RR vs SP vs PP), we especially found significant correlations in the RR phase of the disease. This supports previous observations that suggest that axonal loss already takes place in early phases of the disease (Chard et al., 2002). Strikingly, in this phase of the disease the anti-NfL-index is significantly correlated to all three MRI measures that have been shown to reflect axonal damage (both atrophy measures and the T1-hypointense lesion load) whereas there is no significant relation to the T2 lesion load, a measure that is much more pathologically heterogeneous. In a recent longitudinal study, we found that although

brain atrophy was greater in later phases of the disease, the rate of development of atrophy was greatest in younger patients with a short duration of symptoms (Kalkers et al., 2002). The detection of abnormal anti-NfL indices in patients with RR disease is consistent with recent reports that such patients may develop axonal damage or derangements of neurofilament protein early in the disease (Bjartmar et al., 2001). The absence of significant correlations between CSF findings and MRI measurements in the progressive phase of the disease (SP and PP) might be explained by the small numbers of subjects being involved. On the other hand, our results appear to differ from an earlier report using the same methodology that found elevated anti-NfL and and to lesser extend NfH indices in patients with PP and SP MS (Silber et al., 2002). The most likely explanation for these differences lies in the selection of study objects. In the earlier study CSF was collected when LP were performed as diagnostic procedures. In the RR subjects this was usually related to a relapse or soon thereafter and in the progressive groups the indication for CSF analysis was usually a recent deterioration in clinical condition. In the PP subjects this was often when disability was becoming more apparent and in the SP subjects at the transition between a RR course and disease progression (a median of about 12 years). It has been suggested that during this transitional phase of early disease progression there is a process of matrix destruction. If this were the case, it is likely that axons would be exposed, therby augmenting a humoral response to these proteins. In contrast, in the current combined study, the subjects were volunteers and were more likely to have had a stable course in the preceding months. The duration of symptoms was considerably longer in both the SP and PP groups than in the initial series and in the majority there was a long lapse between CSF sampling and the last relapse, with a median of 77 months in the SP group. It is thus likely that the disease was more quiescent in subjects in this series with less ongoing matrix destruction.

The current study is consistent with the previous in that there seems to be some degree of selectivity in the humoral response: correlations with the previous one in that there seems to be some degree of selectivity in the humoral response: correlations of MRI measures with the anti-NfH index were weaker and a correlation with the IgG index was absent.

Even though our findings strongly suggest that antibodies to NfL are produced during the course of the disease in relation to ongoing tissue damage, our data give no answer to the question of whether these antibodies have a pathogenic role in relation to

axonal damage or rather are phenomenon secondary to release of increased amounts of axonal proteins.

Although our assay was similar to that used by others (Lycke et al., 1998), we were not able to detect NfL in CSF. Similarly, colleagues working independently were able to detect CSF NfL in low levels with no differences between the patientss with RR and progressive MS and other neurological and normal controls (Silber et al., 2002). Several explanations are possible. First, NfL levels in our patients were low, as NfL may be released mainly during relapses, but returns to baseline levels within two months (Lycke et al., 1998). Second, NfL is prone to protease digestion, whereas phosphoryylation protects NfH (Goldstein et al., 1987). Next as pointed out recently, the assay may not be sensitive enough. Finally, anti-NfL antibodies may bind with high affinity to the released NfL thereby preventing detection in the assay.

Traditionally MRI-pathology correlates can only be studied on biopsy specimens (which by definition have been obtained from patients who have atypical presentation and therefore might not represent typical findings of MS) or on autopsy tissue. We propose that our study, correlating MRI parameters to specific biologic markers, provides an *in vivo* MRI-pathology correlation that has the important advantage that it can be longitudinally validated. In view of the growing understanding of the contribution that axonal damage leads to clinical disability and the belief that therapeutic intervention should begin early in the disease course to diminsh disability, both MRI and CSF markers of axonal destruction in MS are of great potential clinical relevance. If proven to be real surrogates, either alone or in combination, they may assist clinicians in guiding therapeutic decisions by identifying patients at risk for developing a rapidly progressive disease course.

Chapter 3.3

Axonal damage accumulates in the progressive phase of multiple sclerosis: three year follow up study

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Abstract

Neurofilament phosphoforms (Nf) are principal components of the axoskeleton released during axonal injury. Cerebrospinal fluid (CSF) levels of Nf phosphoforms might be useful surrogate markers for disability in multiple sclerosis (MS), aid in distinguishing clinical subtypes, and provide valuable prognostic information.
Thirty-four patients with MS were included in a three year follow up study along with 318 controls with other non-inflammatory neurological diseases. CSF levels of two Nf heavy chain (NfH) phosphoforms (NfH^{SMI35}, NfH^{SMI34}) were quantified at baseline and three year follow up using new ELISA techniques. Levels of NfH phosphoforms, the degree of phosphorylation (NfH^{SMI34}: NfH^{SMI35} ratio), and changes in NfH levels between baseline and follow up (ΔNfH) were related to the clinical phenotype (RR or SP/PP), to three clinical scales (Kurtzke's EDSS, ambulation index (AI), and nine hole peg test (9HPT)), and to progression of disability.
A significantly higher proportion (59%) of patients with SP/PPMS experienced an increase in NfH^{SMI35} levels between baseline and follow up compared with those with RRMS (14%, $p<0.05$). CSF NfH^{SMI34} levels at baseline were higher in patients with SP/PP (11 pg/ml) compared with RR (7 pg/ml, $p<0.05$) and NfH^{SMI35} levels were higher at follow up in SP/PP (129 pg/ml) compared with levels below assay sensitivity in
RR ($p<0.05$). NfH^{SMI35} correlated with the EDSS ($r_s = 0.54$, $p<0.01$), the
AI ($r_s = 0.42$, $p<0.05$), and the 9HPT ($r_s = 0.59$, $p<0.01$) at follow up. The increase in NfH during the progressive phase of the disease together with the correlation of NfH^{SMI35} with all clinical scales at follow up suggests that cumulative axonal loss is responsible for sustained disability and that high NfH^{SMI35} levels are a poor prognostic sign.

Introduction

Axonal pathology remains the "Achilles heel" of neurology. New insights from recent studies into the "axonal death cascade" (Waxman, 2003), in multiple sclerosis (MS) are that a high number of transected axons are already present in acute lesions (Trapp et al., 1998; Fergusson et al., 1997), independent of demyelination (Bitsch et al., 2000), in patients with a short clinical course (Bjartmar et al., 2001; Trapp et al., 1998) and as a result of electrical activity in a hostile microenvironment (Kapor et al., 2003). Axonal loss results in atrophy of the spinal cord (Losseff et al., 1996a), cerebellum, (Losseff et al., 1996b) and cortex (Ge et al., 2001) all of which correlate with disability (Losseff et al., 1997 a,b; Ge et al., 2001). *In vivo* quantification of axonal damage is a key tool for monitoring and understanding axonal pathology in complex diseases such as MS. Neurofilaments (Nf) constitute a major component of the axoskeleton and are promising candidates for quantification of axonal damage because axonal transection results in disintegration of the distal axon membrane and Nf breakdown (Zhai et al., 2003; Griffin et al., 1995). Nf are released into the adjacent compartment—that is, the cerebrospinal fluid (CSF), where they can be measured (Lycke et al., 1998; Semra et al., 2002). This prospective study was stimulated by three questions (McDonald, 2000; Waxman, 1998).

- Can clinical subtypes of MS be distinguished on the basis of axonal damage, (disease heterogeneity; Lucchinetti et al., 2000)?
- Does disability correlate with markers of axonal pathology?
- Can we predict loss of function by using biomarkers for axonal injury?

Methods

The local ethics committees approved the present study, and written informed consent was obtained from all patients.

Patients

A total of 34 patients from a previously reported cohort (Axel et al., 2002; Eikelenboom et al., 2003) with clinically definite MS (McDonald, 2001) were followed up after three years. A second CSF sample was available for 29 patients at the time of study. The patients with MS were classified as having relapsing remitting (RR, n = 11), or progressive (n = 23, SP/PP) disease (Lublin and Reingold, 1996). For the CSF analysis patients with primary (PP) and secondary (SP) progressive disease were pooled because of small numbers. However, we will also present a detailed subgroup analysis for classification of patients with RR (n = 10), SP (n = 16) and PP (n = 3) MS at baseline and at follow up. Nine patients had been started on treatment with interferon beta (IFNß) since recruitment in 1996. The control group consisted of 318 patients with other non-inflammatory neurological diseases (OND) from the National Hospital of Neurology and Neurosurgery, London, UK. Restricted sample volume meant not all assays could be performed on each sample and the numbers available for each comparison are presented in **Table 1**.

Clinical assessment

All patients were assessed within one week of each lumbar puncture using: The Expanded Disability Status Scale (EDSS) (Kurtzke, 1983): ranging from 0 (normal) to 10 (death due to multiple sclerosis). An ambulation index (AI): ranging from 0 (no impairment) to 9 (restricted to wheelchair without independent transfer).
The nine hole peg test (9HPT) measuring upper limb motor function.(Kalkers et al., 2001). The patients were classified as clinically advancing if they worsened on the EDSS scale by at least 1 point for an EDSS < 5.5 or at least 0.5 point for an EDSS ≥ 5.5.

Table 1.

Baseline characteristics of the patients

	OND			Multiple sclerosis		
	NfH^{SMI35}	NfH^{SMI34}	Ratio [a]	All	SP/PP [c]	RR
Number	271	119	60	34	23	11
Male:Female	155:116	61:58	23:28	15:19	11:12	4:7
Age (yrs) [b]	44.0 (1–77.9)	45.4 (34.9–52.9)	47.2 (42.5–51.5)	46.5 (42.5–51.5)	48.5 (42.5–51.5)	39.6 (34.9–47.7)
Disease duration (yrs) [b]	NA	NA	NA	14.0 (8.0–19.9)	16.0 (11.8–21.8)	8.1 (3.7–13.0)
Relapse free interval (monts) [b]	NA	NA	NA	38.0 (8.0–96.0)	83.0 (23.5–144)	8.0 (3.0–38.0)

NA=not applicable; NfH^{SMI34}=NfH detected with SMI34 antibody; NfH^{SMI35}=NfH detected with SMI35 antibody; OND=other neurological diseases; PP=primary progressive; RR=relapsing remitting; SP=secondary progressive, yrs=years

a) The "ratio" of NfH^{SMI34} to NfH^{SMI35} was available for only a subgroup of patients due to restricted sample volume;
b) Values are median (interquartile range);
c) Patients with SP/PP disease had a longer disease duration ($p<0.05$) and relapse free interval ($p<0.05$);

Assays

Samples of CSF were obtained by routine lumbar puncture. Aliquots of CSF were stored at –70 °C until assayed. Levels of NfH phosphoforms were quantified using an in-house enzyme linked immunosorbent assay (ELISA) technique based on commercially available antibodies (Petzold et al., 2003). This ELISA has been optimised for the capture antibody SMI35 that recognises a range of NfH phosphoforms (170 kDa, pI 6.2 to 210 kDa, pI 5.1). In contrast, the capture antibody SMI34 only recognises extensively phosphory-lated NfH (Sternberger and Sternberger, 1983). Unfortunately non-phosphorylated Nf is susceptible to proteases (Sternberger and Sternberger, 1983; Goldstein et al., 1987; Pant, 1988), of which the CSF is a rich source. For this reason NfH^{SMI32} was not measured in the present study and a ratio of NfH^{SMI34} to NfH^{SMI35} was used to approximate the phosphorylation status (see below). Albumin in CSF and serum was determined by standard Laurell "rocket" electroimmunoassay.

Data analysis

All statistical analyses and graphs were done using SAS software (version 8.2, SAS Institute, Inc, Cary, NC). Because of non-Gaussian distribution, median values and the 25–75% interquartile ranges (IQR) were calculated. Independent variables were compared using the non-parametric two sample exact Wilcoxon's rank sum test. If significance was based on small numbers the results were checked by the one tailed Fisher's exact test. The linear relationship between continuous variables was evaluated using the Spearman correlation coefficient. Multiple correlations were corrected using the Bonferroni method. Linear regression analysis was performed using the least squares method.

The change in NfH levels between baseline and follow up was expressed as the difference:

$$\Delta NfH = NfH_{follow\ up} - NfH_{baseline}.$$

A positive number indicated an increase in the NfH level at follow up. Because the interassay coefficient of variation for NfH is 10.6%, only an increase of at least 11% was considered for further statistical analysis (Petzold et al., 2003).

The phosphoform ratio is an estimate of the degree of phosphorylation and was expressed as a cross-sectional measure:

$$\text{Ratio} = (NfH^{SMI34} / NfH^{SMI35})x10$$

A decrease in the ratio indicated an overall reduction in the level of phosphorylation. Values with zero denominators (or NfH at baseline and follow up below assay sensitivity) could not be used for calculations and were excluded from this analysis.

Results

The demographic data at baseline are shown in **Table 1**. As expected at baseline, EDSS, AI, and 9HPT were worse in patients with SP/PP disease than in those with RR disease. The CSF levels of $NfH^{SMI35}{}_{baseline}$ were higher in patients with OND than in those with MS ($p<0.001$, **Table 2**). The CSF levels of $NfH^{SMI34}{}_{baseline}$ were similar in both groups. No correlations were found between the Nf phosphoforms or their ratio and age, disease duration, time from last relapse, relapse frequency, or the CSF albumin:serum albumin ratio (data not shown). The shortest relapse free time was three months at baseline in two patients. At follow up one patient with SP/PPMS experienced a superimposed relapse two weeks prior to the CSF sampling. There was no correlation with time from relapse in patients with SP/PPMS or RRMS at either sampling point

Axonal damage accumulates in SP/PP disease

A significant increase in NfH^{SMI35} from baseline to follow up was observed in a higher proportion of patients with SP/PPMS (13/22, 59%, **Figure 1**), when compared with patients with RRMS (1/7, 14%, $p<0.05$). At follow up the median CSF level of NfH^{SMI35} was higher for patients with SP/PP disease compared with patients with RR disease (see **Table 2**, $p<0.05$).

An increase of NfH^{SMI34} levels was observed in a similar proportion of patients with RR (5/7, 71%) and with SP disease (15/22, 68%). The CSF levels of $NfH^{SMI34}{}_{baseline}$ were higher in patients with SP/PP than with RR disease (**Table 2**, $p<0.05$). The proportion of patients with RR (5/7, 71%) in whom NfH^{SMI34} increased was higher than the proportion with an increase in NfH^{SMI35} (1/7, 14%, $p<0.05$). The NfH^{SMI34}: NfH^{SMI35} ratio decreased in 6/7 (86%) of patients with RR and 12/22 (55%) with SP disease. Neither of these comparisons reached statistical significance.

Table 2.

CSF levels and ratio of NfH phosphoforms, EDSS, AI, and 9HPT at baseline in patients with MS and OND (controls), and CSF levels and ratio of NfH phosphoforms, EDSS, AI, and 9HPT, and change over time (ΔNfH) at follow up in patients with MS. Values are median (interquartile range)

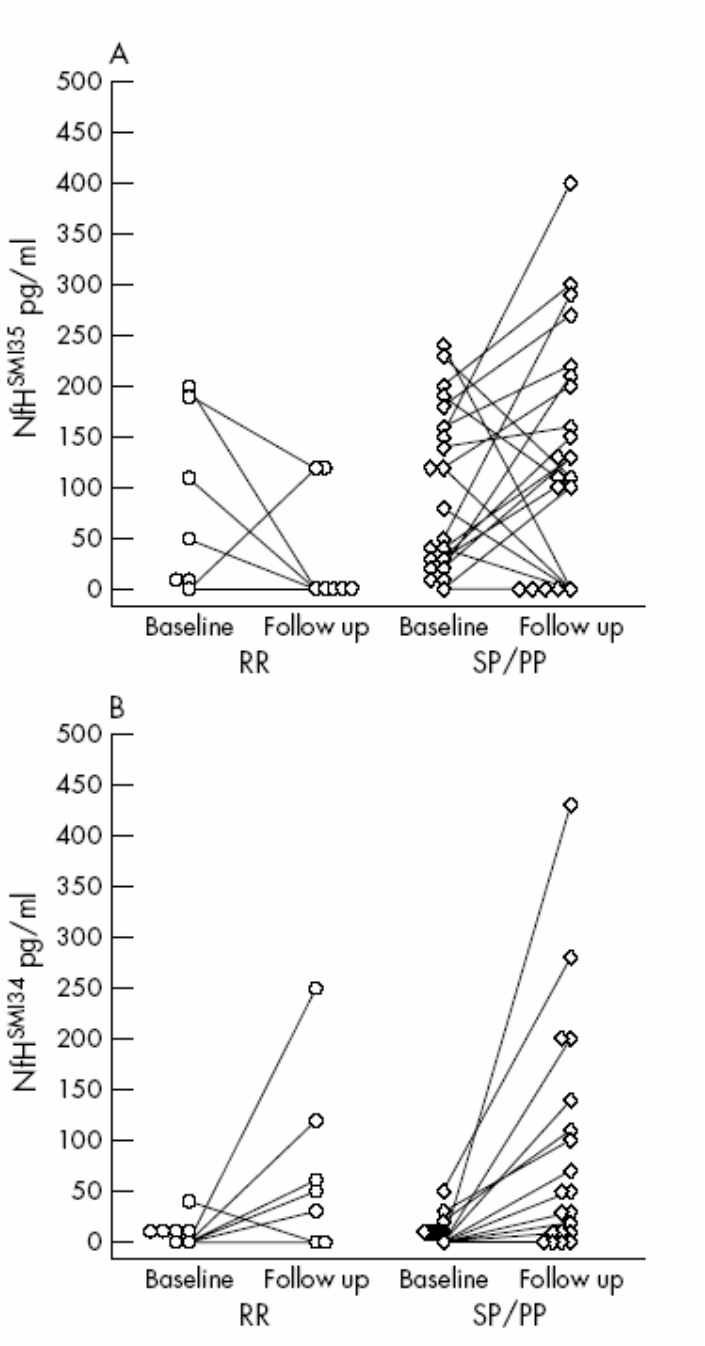
		Multiple sclerosis		
	OND	All	SP/PP	RR
Baseline				
Number	217/119	29	19	10
NfH^{SMI35} (pg/ml)	260 (0–3990) [a]	78 (80–610)	95 (25–163)	53 (11–139)
NfH^{SMI34} (pg/ml)	10 (0–12000)	9 (7–13)	11 (7–14) [c]	7 (5–9)
Ratio	1.52 (0.36–5.49)	1.5 (0.6–5.4)	1.7 (0.6–5.8)	1 (0.42–3.5)
EDSS	N/A	3.25 (2.0–6.5)	6.0 (3.0–7.0) [a]	1.5 (1.0–2.0)
AI	N/A	2 (1–7)	6 (2–8) [b]	1 (1–1)
9HPT	N/A	24.5 (20.5–28.5)	26.0 (24.0–30.0) [b]	20.0 (18.0–22.0)
Follow up				
Number		29	19	10
NfH^{SMI35} (pg/ml)	N/A	113 (0–178)	129 (0–209) [c]	0 (0–120)
NfH^{SMI34} (pg/ml)	N/A	50 (9–129)	30 (10–114)	51 (3–120)
Ratio	N/A	3 (1–10.1)	3 (1–10.1)	5 (0–10)
ΔNfH^{SMI35} (pg/ml)	N/A	4 (–59–98)	82 (–38–115)	–49 (–38–104)
ΔNfH^{SMI34} (pg/ml)	N/A	37 (–1–123)	22 (–3–95)	51 (1–112)
EDSS	N/A	4.5 (3.5–6.0)	5.5 (4.0–6.5) [b]	3.0 (2.5–4.0)
AI	N/A	2 (2–5)	4 (2–7) [b]	1 (1–2)
9HPT	N/A	23.5 (20.0–28.5)	24.5 (21.5–31.0) [c]	20.0 (18.0–21.0)

9HPT, nine hole PEG test; AI, ambulation index; EDSS, Expanded Disability Status Scale; MS, multiple sclerosis; N/A, not applicable; NfH^{SMI34}, NfH detected with SMI34 antibody; NfH^{SMI35}, NfH detected with SMI35 antibody; OND, other neurological diseases; PP, primary progressive; RR, relapsing remitting; SP, secondary progressive.

a) level of significance, $p<0.001$;
b) level of significance, $p<0.01$;
c) level of significance, $p<0.05$

Figure 1.

A CSF NfH^{SMI35} levels and
B. CSF NfH^{SMI34} levels in patients with relapsing remitting (RR) and secondary/primary progressive (SP/PP) forms of multiple sclerosis (MS). A significantly higher proportion of patients with SP/PPMS (13/22) had an increase in CSF NfH^{SMI35} levels between baseline and follow up (straight lines) when compared with patients with RRMS (1/7, $p<0.05$, Fisher's exact test)



Axonal injury correlates with disability

NfH^{SMI35} levels correlated with all three clinical scales at follow up (**Figure 2**). The correlation was strongest for the 9HPT (r_s = 0.59, p = 0.001), followed by the EDSS (r_s = 0.54, p <0.01), and the AI (r_s = 0.42, p<0.05). The correlation with the AI was lost after Bonferroni correction. One outlier was observed for the 9HPT (fig 2C), but its exclusion did not change the significance of the correlation (r_s = 0.55, p<0.01). The NfH phosphoform ratio correlated with the EDSS (r_s = 0.52, p<0.05) at follow up, but this significance was lost after the Bonferroni correction. No significant correlation was found between either NfH phosphoform and the change in EDSS, AI, or 9HPT over the three-year period (data not shown). At baseline, no such correlations were found after Bonferroni correction for either of the NfH phosphoforms or their ratio.

Axonal injury and prognosis

The median CSF NfH^{SMI35} level (107 pg/ml) of patients with MS who progressed on the EDSS scale within three years showed a tendency to be higher compared with the median level of those who remained stable (38 pg/ml). However, this difference did not reach statistical significance for either the total MS cohort or the clinical subtypes **(Table 3)** However, using an arbitrary cut-off level of 20 pg/ml (assay sensitivity) on the baseline cohort, the positive predictive value of high NfH^{SMI35} levels for predicting progression of patients on the EDSS scale within three years was 100% for RRMS and 20% for SP/PPMS with specificity of 100% and 20% and sensitivity of 87.5% and 75%, respectively.

Three patients who had RR disease converted to SP disease in the three year observation period. These patients had a higher median CSF $NfH^{SMI35}_{baseline}$ level (123 pg/ml) when compared with non-converting RR patients (49 pg/ml). Again this difference did not reach statistical significance.

Patients treated with IFNß had a lower median EDSS at baseline (2.0 *v* 5.5; p<0.05) and follow up (4.0 *v* 4.5; p<0.05) and were less progressive (improvement by a median of 1.5 points on the EDSS *v* no change; p<0.05) compared with untreated patients. The patients treated with IFNß had lower CSF $NfH^{SMI34}_{baseline}$ levels (7 pg/ml) compared with non-treated patients (11 pg/ml, p <0.01). No such differences were found for $NfH^{SMI35}_{baseline}$ or the NfH phosphoform ratio.

Figure 2.

Correlation between the CSF NfHSMI35 levels and
(A) the Expanded Disability Status Scale (EDSS),
(B) the ambulation index (AI), and
(C) the 9HPT (nine hole peg test; log transformed scale) at follow up

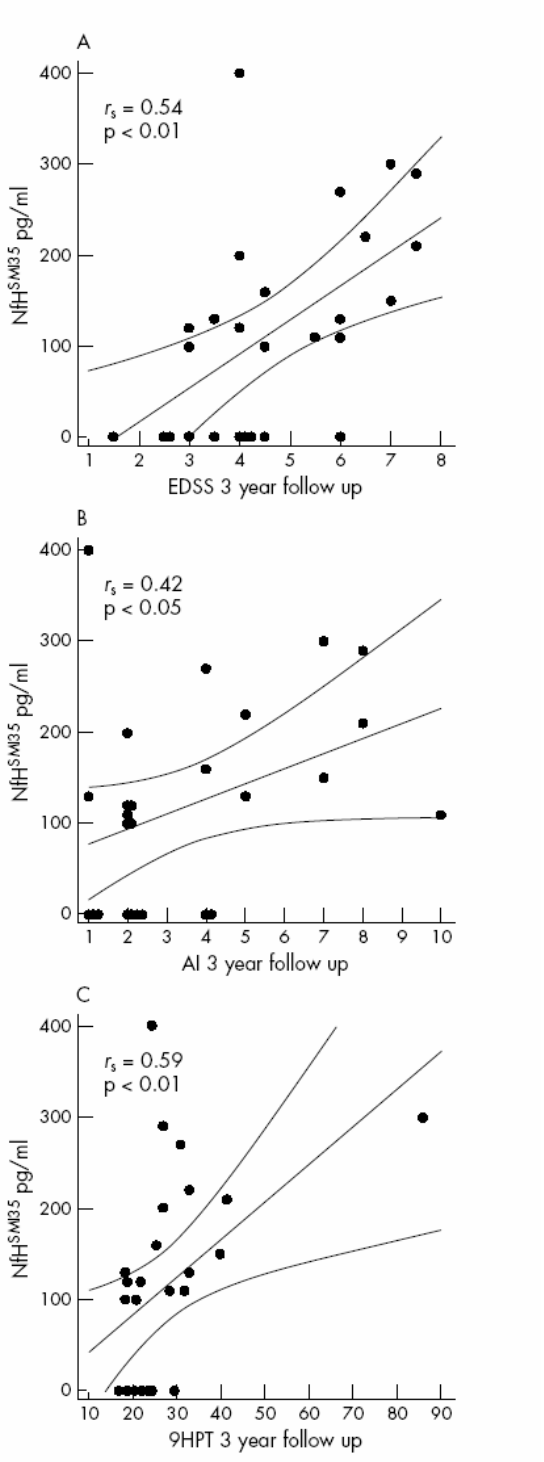


Table 3.

CSF levels and ratio of NfH phosphoforms for clinically progressive versus stable patients at baseline. Values are median (interquartile range)

	MS (all patients)		SP/PP		RR	
	Stable	Progressive	Stable	Progressive	Stable	Progressive
Number	19	15	16	7	3	8
NfH^{SMI35} (pg/ml)	38 (17-155)	107 (49-163)	42 (24–173)	104 (78–173)	5 (0–53)	115 (43–165)
NfH^{SMI34} (pg/ml)	11 (5-13)	8.0 (5-14)	11 (7.5–13.0)	9.0 (5–16)	2 (0–5)	7.5 (6–11)
Ratio	32 (0.6-5.8)	1.0 (0.6-5.8)	3.2 (0.6–6.0)	1.1 (0.5–1.7)	2.5 (0.9–4.0)	0.9 (0.4–3.5)

PP=primary progressive; RR=relapsing remitting; SP=secondary progressive

Subgroup analysis

Since there are differences in pathogenesis between SPMS and PPMS, particularly with reference to the degree of inflammation, CSF levels of NfH^{SMI35}, NfH^{SMI34}, and their ratio were also examined for all individual subgroups.

At baseline, there was a significant difference between these groups for NfH^{SMI34} ($F_{2,26} = 5.00$, $p<0.05$). The post hoc analysis revealed that CSF NfH^{SMI34} levels were higher in patients with PP disease (mean 26.6 pg/ml) compared with patients with SP (mean 10.19 pg/ml, $p<0.01$) or RR disease (mean 9.30 pg/ml, $p<0.01$). At follow up no such difference was found, probably due to the small numbers and therefore no post hoc analysis was performed.

Discussion

The present study provides evidence that accumulation of axonal damage as estimated by serial CSF NfH^{SMI35} levels predominates in SP/PPMS reveals a correlation between CSF levels of NfH^{SMI35} and the degree of disability on three clinical scales (EDSS, AI, 9HPT), failed to demonstrate that CSF NfH phosphoforms might predict the development of new disability in patients with MS.
We interpret these findings on the basis of the epidemiologically supported hypothesis that axonal damage is a gradual cumulative process during the course of the disease (McDonald, 1992; Pant, 1988) and that loss of neurological function is a direct consequence of axonal injury (McDonald, 2000; Trapp et al., 1998, Waxman 1998).

Firstly, NfH^{SMI35} levels increased from baseline to the three year follow up sampling in about half of our patients with MS. A significantly higher proportion of these patients had SP/PP than RR disease. Additionally, the median $NfH^{SMI35}{}_{\text{follow up}}$ level was significantly higher in SP/PP rather than in RR disease. The marked increase of NfH^{SMI34} levels suggests that NfH phosphorylation may increase with disease duration. This interpretation contrasts with the consistent immunocytochemical observation that injured and demyelinated axons stain for non-phosphorylated NfH (NfH^{SMI32}) (Geurts et al., 2003; Gilgun-Sherki et al., 2003; Werner et al., 2001; Pitt et al., 2000; Trapp et al., 1998). However, none of these studies presented quantitative data comparing the number of axons staining for phosphorylated versus non-phosphorylated NfH. A further complicating factor is that proteolytic enzyme activity is a prominent feature of the MS plaque (Cuzner, 1978; Adams, 1975) and potentially affects the levels, particularly of non-phosphorylated NfH, which is susceptible to proteolysis (Sternberger and Sternberger, 1983; Goldstein et al., 1987; Pant, 1988). To address this question we are currently analysing the quantitative distribution of NfH phosphoforms (NfH^{SMI32}, NfH^{SMI34}, and NfH^{SMI35}) in microdissected brain tissue homogenates from a previously published cohort (Gveric et al., 2003 and 2001; Petzold et al., 2002). An increase in NfH phosphorylation supports our finding and can be explained by targeted phosphory-lation of the KSP repeats of the NfH and NfM tail domains by ERK1/2.(Grant and Pant, 2000). Fibrin upregulates ERK1/2 (Akassoglou et al., 2002) and has shown to be deposited on injured axons (Gvericet al., 2003 and

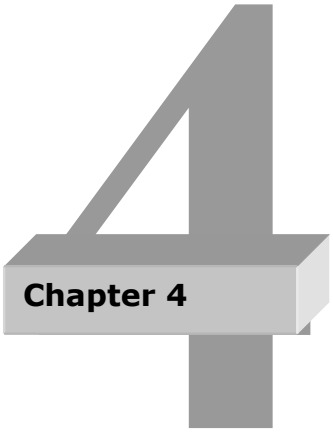
2001; Akassoglou et al., 2000). Additionally the mitogen activated protein (MAP) kinases SAPKs and ERK1/2 are activated by glutamate (Brownlees et al., 2000; Schwarzschild et al., 1999 and 1997; Xia et al., 1996; Kurino et al., 1995), which in turn leads to Ca influx, slowing of axonal Nf transport, and increased Nf phosphorylation. (Ackerley et al., 2000 and 2003). Glutamate toxicity is an important pathological feature in MS, metabotropic glutamate receptor group I alpha is upregulated on axons in MS (Geurts et al., 2003) and experimental treatment with the AMPA/kainate antagonist NBQX reduces axonal damage in experimental autoimmune encephalomyelitis (Pitt et al., 2000). The present CSF results are also consistent with the postmortem observation that axonal damage increases with time in SP/PPMS patients (Kuhlmann et al., 2002), with additional support from brain imaging (Barkhof, 2002) and epidemiological studies (Confraveux et al., 2000).

Secondly, we found a correlation between CSF NfH^{SMI35} and three clinical scales. This finding indirectly confirms two previous reports on a different Nf subunit (the 68 kDa light chain, NfL) (Semra et al., 2002; Lycke et al., 1998). Lycke et al. (1998) reported CSF NfL correlated with the EDSS at baseline ($r = 0.27$) and follow up ($r = 0.34$) in patients with RRMS. Semra *et al.* (2002) found CSF NfL correlated with the EDSS
($r = 0.41$) in patients progressive MS. However, no such correlations were found at baseline in our original study (Eikelenboom et al., 2003), the present follow up cohort, or a recent study on patients with RRMS and SPMS Malmestrom et al., 2003). Because clinically, some of our patients with SP/PP disease improved a degree of disability at baseline, it is likely that conduction block and demyelination which are, in contrast to axonal loss, reversible, contributed to the deficit.

Thirdly, a tendency for higher median CSF $NfH^{SMI35}_{baseline}$ and $NfH^{SMI34}_{baseline}$ levels were observed in those patients with MS who progressed clinically on the EDSS scale. This was most marked for patients with RR disease and suggests that axonal damage during the course of MS is a poor prognostic feature. However, the study failed to show statistical difference. The high positive predictive value and sensitivity suggest that this might be due to the small sample size. It is important to note that axonal loss is not the dominant pathological feature in MS compared with other neurological diseases (Petzold et al., 2002 and 2003). Nevertheless, the demonstrated slow

accumulation of axonal loss seems a logical explanation for the development of sustained disability in the progressive course of the disease.
When interpreting the present data one needs to consider that the patient group is population based and the numbers small. Clinically, there was no significant change in the EDSS of the patients with SP/PPMS within three years. This represents a benign course compared with the more rapid progression observed in other cohorts of patients selected from hospital populations. Additionally the low median CSF NfH^{SMI35} level of the RRMS cohort at follow up would suggest that these patients might have a benign disease course. The results must be interpreted with caution and will need to be cross validated in other longitudinal studies with different cohorts of patients.
Taking all these observations together, the results of the present study are in accordance with the current concept of progressive axonal degeneration in MS which is based on evidence from animal (Petzold et al., 2003; Pryce et al., 2003), human postmortem (Gveric et al., 2003; Kuhlmann et al., 2002; Bjartmar et al., 2001; Kornek et al., 2000; Trapp et al., 1998, Ferguson et al., 1997), magnetic resonance spectroscopy (van Waesberghe et al., 1999), magnetic resonance imaging (Ge et al., 2001; Losseff et al., 1996 a & b; Truyen et al., 1996) and epidemiological studies (Confraveux et al., 2000, Runmarker et al., 1993).

In conclusion, the findings of our prospective three-year study support the idea that CSF NfH phosphoforms might be valuable surrogate markers, which have the potential to be used as new secondary outcome measures in trials of MS treatment.



Summary and conclusions

Summary and conclusions

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the human central nervous system. Demyelination and axonal loss in brain and spinal cord impair nerve conduction and lead to neurological disability. Although MS has been extensively studied, the etiology of the disease is still unknown. Various histopathological patterns and different clinical courses have been described, indicating that MS may be hetero-geneous with respect to its pathogenesis. Genetic and environmental factors seem to determine disease susceptibility. It is widely believed that MS is an autoimmune disease, initiated by activation of CD4+ autoreactive T cells and their differentiation into a Th1 phenotype. Damage of the target tissue, the central nervous system, is most likely mediated by other components of the immune system, such as antibodies, complement, CD8+ T cells, and factors produced by innate immune cells (i.e. glutamate). The inflammatory reaction is associated with the formation of plaques of demyelination with relative axonal sparing. However, there is also diffuse damage in the normal appearing white matter, which is characterized by axonal loss and microglia and astroglia activation. This suggests that a neurodegenerative process plays a role, next to the inflammation. The timing of these processes (inflammation and neurodegeration) in relation to the disease stages is still to be determined. More insight in these processes in the different subgroups of patients will hopefully lead to better treatment.

The aim of this thesis was to further investigate the role of several indicators of various processes in MS. We therefore looked at the associations between immunological biomarkers and biomarkers reflecting damage of the central nervous system on the one hand and clinical and imaging parameters on the other hand.

The studies, described in **chapter 2**, address relations between immune measurements and clinical and disease course as well as (long-term) disease progression on MRI. In **chapter 2.**1 a longitudinal study was performed to investigate the expression of chemokine receptor expression (CCR5 and CXCR3) on T cells in patients in 124 MS patients with different clinical subtypes of the disease. Higher percentages of CD8 positive cells were found in SP compared to PP subjects. PP patients showed also a

higher percentage of CCR5 expressing CD8 positive cells compared to RR patients. When the two progressive subtypes were taken together and compared to the RR patients, it appeared that progressive patients had a significantly higher percentage of CD8 cells expressing the receptor CCR5. The predictive value of CXCR3 expression on CD8 positive cells in the total group, CXCR3 expression on both CD4 and CD8 positive cells (in SP), as well as CCR5 expression on CD8 positive cells (in RR) for the annualized change in T2 lesion load (LL) was confirmed. The findings of **chapter 2.1** show that the two ligand /receptor pairs CCL5/CCR5 and CXCL10/CXCR3 play a role during active disease by showing that expression of CCR5 and CXCR3 on T lymphocytes predicts future disease activity as documented with MRI. Our finding of significant correlations of chemokine receptor expression with change in T2 lesion load (representing new lesions) and not with T1 lesion load (representing more destructive lesions only) suggests that the chemokine axis is more relevant with respect to initiation than to long-term outcome of lesions.

In **chapter 2.2** a cross-sectional study on CXCL10 and CCL2 in blood and CSF is described in 51 MS patients, these markers were related to clinical and MRI measurements of disease state. We observed no differences in chemokine expression between the subgroups and also no significant correlations could be detected between chemokine levels and the clinical (EDSS, disease duration, age and time since last relapse) and MRI data (T2 and T1 hypointense and Gadolinium enhancing lesion load and the athrophy measurements, VF and PF). In conclusion, in clinically stable MS patients, CXCL10 and CCL2 as measured in blood or serum are not likely to be useful markers of disease activity in MS.

In **chapter 2.3** a longitudinal study was performed to investigate whether expression of certain adhesion molecules (VLA-4, LFA-1, ICAM-1) on T lymphocytes differs between subtypes of MS and predicts disease activity with respect to future lesion development on T1 and T2-weighted MRI scans in 124 MS patients. Differences were found between the clinical subgroups for CD11a and CD18 expression on T cells, as well as CD49d+ CD8+cells and CD29+ T cells. When looking at the demographics, relations were found in the CD8+ cells for CD49d+ and CD29+ cells with age, EDSS and disease duration. The disease progression on MRI (delta T2 LL) correlated with the following adhesion molecules: CD29 in CD4+ cells, CD18 in CD4+ cells and CD8+ cells and CD49 in CD4+ cells. Strikingly these correlations were stronger in the SP population. Looking at the delta T1 lesion load in RR MS patients a significant

correlation was found with CD49d in CD4+ cells. These finding show that especially the integrins play an important role in the induction phase of lesion development in MS.

In **chapter 2.4** in a cross-sectional study design, sex differences in MS patients (n=124) and controls (n=34) were revealed between expression of cytokines in both CD4+ and CD8+ T cells. No differences were seen between males and females in the total MS group. Compared to males, female patients had higher pro-inflammatory cytokine levels in the progressive phase of the disease and lower levels in the relapsing phase of the disease. This might implicate that cytokine production, and sex differences in cytokine production might differ between disease phases, probably related to underlying disease mechanisms.

In **chapter 2.5** we investigated whether opticospinal MS (OSMS) differs from classic MS (CMS) with respect to immune markers expressed by peripheral T cells. Compared to CMS we observed lower percentages of the CD4+ T cells producing the Th1 cytokine IFN γ and higher percentages of CD8+ T cells producing the Th2 cytokine IL10 in OSMS. These findings indicate a shift towards Th2, which is line with other concepts hypothesized regarding to Devic's disease (neuromyelitis optica; Lucchinetti et al., 2002; Lennon et al., 2004).

In **chapter 3** of this thesis, biological markers involving degeneration of the central nervous system are applied to reveal clinical and paraclinical subgroups.

In **chapter 3.1** we investigated the relationship between the concentration of biomarkers for glial reaction in the CSF of 51 MS patients of the different clinical subtypes and their degree of disability and validated these findings with a postmortem study. We observed a trend for increasing S100B levels from PP to SP to RR, whilst ferritin levels were higher in SP than in control patients. The S100B: ferritin ratio discriminated patients with RR MS from SP, PP or control patients. MS patients with poor ambulation or severe disability had significantly higher CSF GFAP levels than less disabled MS or control patients. The post-mortem study showed significantly higher S100B levels in the acute than in the subacute plaques, whilst ferritin levels were elevated in all MS lesion stages. In conclusion, S100B may be associated with the relapsing phase of the disease (confirmed by post-mortem observation) as opposed to ferritin, which is elevated throughout the entire course. GFAP correlated with disability scales and may therefore be a marker for irreversible damage.

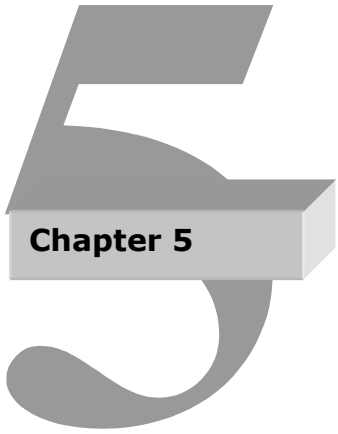
In **chapter 3.2** we evaluated the neurofilaments light (NfL) and heavy chain (NfH) and their respective antibodies in matched CSF and serum samples from patients with

different subtypes of MS and their relation with a variety of MRI parameters of tissue damage. Neurofilaments heavy and light chain, as well as their antibodies could not discriminate between the subpopulations of MS patients. The anti-NfL index (CSF anti-NfL units/serum units)/ (CSF albumin/serum albumin) correlated with all the brain tissue damage markers (parenchymal and ventricular fraction, T1 and T2 lesion load). For the anti-NfH index there was a significant relation with the atrophy marker parenchymal fraction. This study might give a direction for a biological marker, which is related to *in vivo* MRI-pathology.

In **chapter 3.3** a longitudinal study was performed on twenty–eight MS patients and nine controls on CSF levels of the phosphorylated (NfH p) and the extensively phosphory-lated neurofilament heavy chain (NfH ep). Levels of NfH phosphoforms, the degree of phosphorylation (ratio of NfH phosphoforms) and changes of NfH levels between baselines and follow-up was then related to the clinical phenotype and to clinical scales. Seventy percent of the progressive patients experienced an increase in NfH p levels between baseline and follow-up and twenty percent of the RR group. Progressive patients had higher levels of NfH p levels than stable RR MS patients at baseline and follow up. Median NfH p levels at baseline were higher in clinically advancing RR versus stable RR MS patients. NfH p correlated with the clinical scales at follow up. This study demon-strated that CSF Nf phosphoforms are predictors for development of disability in RR MS disease, suggesting that early axonal injury is a poor prognostic sign. During the progressive phase of the disease an increase of Nf was observed suggesting cumulative axonal loss.

Conclusions

- Immunological markers on peripheral blood cells, especially chemokinereceptors (CXCR3 and CCR5) and adhesion molecules (especially integrins), may predict future lesion development
- Gender differences in cytokine production differ between disease phases
- Opticospinal MS seems to be a Th2 mediated disease as was hypothesized for neuromyelitis optica
- Glial activity reflected by S100B and GFAP may help to differentiate subgroups in MS
- Neurofilament antibodies seem to be related to *in vivo* pathology on MRI
- CSF Nf phosphoforms are predictors for development of disability in RR MS disease



Discussion and future directions

Discussion and future directions

There are only very few biomarkers that are accepted as reliable and practicable parameters for determining the nature and state of activity of MS. At present, certain biomarkers are used to complement and confirm clinical, imaging or other findings, but there is no test that convincingly diagnoses MS. Apart from the initial diagnosis, biomarkers may help to monitor the disease course, to classify particular subsets of the disease and, ideally, to select optimal treatment and determine its efficacy over time (Compston et al., 2005). Whilst there is agreement that the identification of immunological biomarkers would be of enormous help for diagnosis, classification, and monitoring of natural course and therapeutic efficiency, emphasis of current research is also shifting towards markers of neurodegeneration.

Neuroinflammation versus neurodegeneration

For many years, the central concept underlying ideas on the pathogenesis of MS has been that the cascade of inflammatory events culminating in demyelination depends on the peripheral activation of autoreactive T cells. According to this analysis, activated T cells express adhesion molecules and chemokine receptors on their surface, allowing them to cross the Blood-brain-barrier and then disperse into the parenchyma. Within the CNS these T cells re-encounter specific antigen and set up an inflammatory process. Recent studies, however, indicate that the pathogenesis is much more complex. Some of these data suggest a primary neurodegenerative process independent from immune-mediated inflammation. However, others strongly believe the inflammatory and degenerative components are inter-related and should not be regarded as fully independent events (Compston et al., 2005).

The studies described in the first part of this thesis are in accordance with the thought that neuroinflammation plays a role especially in the beginning of the disease. The relapses observed in patients seem to reflect flairs of inflammatory activity. These relapses become rare during the later course of the disease, while neurodegeneration continues or even becomes more prominent.

We have shown that immunological parameters, chemokine receptors (CCR5 and CXCR3) and integrins (VLA4) on T cells, play an important role during the induction phase of lesion development (T2 lesion load) and less in the development of tissue destruction (T1 lesion load) (**chapter 2.1** and **2.3**).

Chemokines and adhesion molecules (e.g. integrins) can influence the disease course of MS at many levels. Interest in targeting the transport of immune cells in the treatment of MS accelerated after the publication of the results of the natalizumab trials (humanized monoclonal antibody that blocks the α4 leukocyte integrins). The mechanism of this drugs, decreasing the migratory capacity of immune cells, have been recently also confirmed by showing that there was a diminished VLA-4 expression on circulating immune cells in natalizumab treated patients (Niino et al., 2006). The efficacy of natalizumab and the appearance of unexpected adverse effects underline both the promise and the challenges involved in the modification of leukocyte transport for the treatment of inflammatory diseases.

Analyses of chemokines and chemokine receptors in blood and cerebrospinal fluid and in brain sections from patients with MS have yielded complex data. Most lymphocytes in cerebrospinal fluid are CD4+ memory T cells, in proportions that are significantly higher than in blood. Most of these lymphocytes in cerebrospinal fluid express CXCR3. Such findings suggest that there is surveillance of the central nervous system by CD4+ memory T cells, which patrol the subarachnoid space in search of antigens and return to the blood or the lymph nodes. T cells expressing CXCR3 are readily found in MS lesions near parenchymal vessels. Infiltrating monocytes in the lesions are derived from a sub-population of blood monocytes and express both CCR1 and CCR5. Phagocytic macrophages do not express CCR1 but are CCR5-positive. Altogether these findings suggest that chemokines regulate monocytes and macrophages by governing their departure from the bloodstream into tissues, their migration through lesions, and their effector functions. Interestingly recent publications on immunotreatment in MS patients have revealed that a reduction of CCR5-expressing blood derived T cells could be induced by intravenous methylprednisolon (Elovaara et al., 2006; Wang et al., 2003) but conflicting data are published on IFN beta treated patients (Kivisakk et al., 2003; Teleshova et al., 2002). This further emphasizes the possible involvement of this receptor in the active inflammatory part of the disease.

There are two sides of inflammation, a protective and destructive side (Kerchentstein et al., 2003). It seems likely that in MS there is a disturbance in the dialogue between immune system and nervous system. It probably starts as a time- and site-specific defense mechanism that could later evolve into a destructive and uncontrolled reaction, leading to axonal loss. However, probably axonal loss is a multifactorial process. Inflammation might damage the axon directly or via a different pathway that includes demyelination (Hohlfeld et al., 1997; Bo et al., 1994; Selmaj et al., 1991). A number of cellular and humoral mediators of the immune response have been shown to be capable of damaging axons including T cells, macrophages, antibodies, nitric oxide, glutamate and matrix metalloproteases. Myelin and oligodendrocyte destruction probably precede axonal injury in MS. Oligodendrocyte death can be caused by distal oligodendrocyte process atrophy or primary oligodendrocyte degeneration (e.g. caused by genetic defects or metabolic impairment in oligodendrocytes) (Wingerchuk et al., 2001). Moreover, these processes may differentiate in individual patients and patient groups. Cerebrospinal fluid (CSF) markers might add important information about the ongoing pathological processes. Although CSF research has been mainly focused on the inflammatory response in MS, glial response and axonal damage are gaining interest.

As demonstrated glial activity reflected by CSF S100B and CSF GFAP may help to further identify subgroups with relapsing remitting disease course or more disability respectively (**chapter 3.1**). Other studies confirm our finding of GFAP being a marker of increasing neurological disability (Norgren et al., 2004, Malmestrom et al., 2003, Rosengren 1995). In different subgroups elevated levels were found. In our study we especially found this in SP patients while others reported these findings in RR patients (Rosengren et al., 1995). However, GFAP has reported to be raised in a variety of neurological conditions, such as dementia, traumatic brain injury (Kay et al., 2003; Eng et al., 1994). It certainly is a non-specific marker of brain damage, but might also be regarded as a marker indicating the change from the active (inflammatory) in the more chronic (more neurodegenerative) disease phase.

S100B has been shown to be elevated during relapses (Missler et al., 1997) and might differentiate between secondary progressive and relapsing remitting MS (Jongen et al., 1997), but conflicting results have been published (Malmestrom et al., 2003). In addition, it has been shown that in RRMS patients S100B might differentiate between responders (higher S100B) and nonresponders (lower S100B) in IFN beta treated

patients (Petzold et al., 2004). We confirmed that S100B was significantly higher in RR MS than controls, but only a S100B: ferritin ratio could discriminate between patients with RR MS from SP, PP or control patients (**chapter 3.1**). Further studies on this marker reflecting astroglial activation are warranted to establish its role as a proper biomarker in MS.

In the second part of the thesis, we have also shown that markers of tissue damage in the central nervous system (by neurofilaments, phosphophorms and their antibodies) are related to development of disability and tissue damage as reflected on MRI. Remarkably, we especially found relations between the MRI parameters and intrathecal production in the RR-phase of the disease. Recent reports have shown that elevated levels of CSF NfL and NfH are associated with relapses (Malmestrom et al., 2003) and with baseline enhancing lesion volume (Lim et al., 2005), giving further support to the view that acute inflammation and axonal pathology take place early in the disease and are at least (partly) related.

Neurofilament phosphoforms are gaining more and more interest in recent years. In this thesis we have demonstrated (**chapter 3.3**) that NfH phosphorylation may increase during the progressive phase of the disease. In our recent publication (Petzold et al., 2006) we found a much higher degree of NfH phosphorylation in patients with more severe disease course using the multiple sclerosis severity score (Roxburgh et al., 2005), a new measure of disease course (**chapter 3.3**). Although it seems that neurofilament phosphorylation might be increased in axonal injury, conflicting results have been published also in other neurodegenerative diseases. The precise roll of this phosphorylation in relation with axonal damage in MS has to be further evaluated.

Neurofilament-L antibodies have been demonstrated to be higher in progressive patients compared to the relapsing remitting patients (Silber et al., 2002). This is line with our findings (**chapter 3.2**). We were not able to confirm the previous finding of higher anti-NfL levels in progressive patients, but MRI parameters might give a better reflection of the stage of the disease than the conventional subgroups. This suggested relation between the autoantibodies and atrophy on MRI should be confirmed in other cohorts.

Clinical relevance of biomarkers in MS

Markers for disease diversity

It is of uppermost importance to differentiate subgroups of MS patients. It has been recognized that although patients are all labeled with the diagnosis MS, it is a heterogenous disease with different clinical symptoms, disease course, MRI, immunological and pathological characteristics. The heterogeneous nature of the disease may need different treatment modalities to optimize the influence of the disease course and the costs involved in the drug treatment.

To better classify the disease and to identify the subgroups, a combination of factors is needed, which can be clinical symptoms, MRI and laboratory characteristics. Moreover, it could be that a gender-based approach to MS could provide further benefits for its treatment and management. The fact that for example hormones (including sex hormones) play a role in the immunopathology of the disease will probably influence the susceptibility and disease course of MS patients. Our study on sex differences in MS has revealed remarkable results, indicating that cytokine production may differ between disease phases in male and female patients.

Higher disease prevalence, as well as an overall better prognosis, in women compared to men is observed in MS (Tomassini et al., 2006). This sex dimorphism may be partly explained by the effect of sex hormones on brain damage and repair mechanisms by inflammation. Another identification of subgroups based on the immunodata on peripheral T cells is the opticospinal MS patient. This group of patients resembles MS patients but differs because of only spinal and optical nerve involvement. Therefore, different pathology and underlying immune mechanisms have been hypothesized for this more neuromyelitis optica resembling disease course. We have confirmed the hypothesis that this may be a more Th2 mediated disease (**chapter 2.5**). The recent identification of autoantibody NMO-IgG has helped to further differentiate between these inflammatory demyelinating diseases. It is a specific marker of neuromyelitis optica and binds at or near the blood-brain barrier. It seems to distinguish neuromyelitis optica and optic-spinal multiple sclerosis (especially Asian type) from multiple sclerosis (Lennon et al., 2004). The use of this autoantibody has recently been incorporated into new diagnostic criteria of Devics disease (Wingerchuck et al., 2006).

Biomarkers and treatment

Immunomodulation

The collaborative effort among clinicians and laboratory scientists has led to a number of biomarker study results that have provided new insight in several promising treatment strategies in MS. Immunomodulatory treatment has been widely available for the last decade. Altogether the currently available treatment options are steroids, interferon beta, glatiramer acetate and mitoxantrone. The latter is limited in use due to cardiotoxicity. The past years biomarkers are gaining interest in treatment evaluation. The discovery of antibodies against interferon-beta has led to new insights, while they can appear in a number of patients and neutralize IFN-beta activity. They are called neutralizing antibodies. Clinical, biologic, and immunologic data have demonstrated that they reduce or abolish the therapeutic efficacy of IFN-beta in 10-20% of patients (Bertolotto, 2004). Till now this has been the only consistent relevant biomarker in therapeutic use.

The studies on biomarkers and pathology of MS have led to development of new therapeutic options, while the (most of the time immunological) targets in MS seem to be relevant for the disease progression. Other potential new immunomodulatory treatment may be monoclonal antibodies that target different key playing molecules in the immunesystem. These are Alemtuzumab (Campath-1H), rituximab and natalizumab (Tysabri). Alemtuzumab is an antibody against CD52, which induces T cell depletion. It reduces the number of relapses and number of new Gadolinium enhancing lesions (Coles et al., 1999). Rituximab is an autoantibody to CD20, inducing B cell depletion, which has been demonstrated to be effective in NMO, a primarily B cell driven MS variant (Cree et al., 2005). Natalizumab (Tysabri) is an antibody directed against the alpha4component of the integrin on the lymphocyte, which when activated binds with an adhesion molecule (VLA-4) on the vascular endothelium, a process essential for the docking of immune cells and their translocation across the blood bran barrier. This agent prevents T and B cells entering into the central nervous system. The results of several studies have shown a dramatic reduction of relapse rate and slow progression of disability (Polman et al., 2006 Rudick et al., 2006). Unfortunately, it blocks also non-specific normal T cells to the CNS and this may explain cases of progressive multifocal leukoencephalopathy

reported with this drug (Yousry et al., 2006; Kleinschmidt-DeMasters et al., 2005; Langer-Gould et al., 2005) Chemokine receptors potentially are interesting targets for drugs in development. Chemokines (chemotactic cytokines) influence the disease by directing the movement of circulating leucocytes to sites of inflammation or injury. Unfortunately in practice chemokine receptors are difficult to antagonize. Most of the MS-trials that involve chemokine receptor antagonists are in phase 1 or 2, the chemokine receptors being involved are CCR1 and CCR2 (Charo et al., 2006). While CCR5 antagonist have been extensively studied in clinical trials in HIV, the applicability for MS may soon be come clear.

Interestingly, immunomodulating drugs in MS seem to have a beneficial effect especially in the early phase of the disease. For example in the optic neuritis treatment trial it has been demonstrated that a single 3 day intravenous course of 1 gram methylprednisolone reduced by about 50% the two years risk of conversion to clinically definite MS. These effects minimize after 5 year follow up. Furthermore, patients with the relapsing remitting course have beneficial effects of interferon and glatiramer acetate on relapse rate, which might be associated with a reduction of progression of disability (Goodin et al., 2002). All clinical trials investigating the effect of IFN on disease progression in patients with secondary progressive MS failed to show any consistency in data favouring the use of the agent in this group, both on clinical and brain atrophy characteristics.

Till now, all therapies have been targeting different steps of the autoimmune process and all act in the periphery to modify the properties, activity or trafficking of different autoreactive lymphocyte populations. Perhaps these therapies might not be very applicable to the rare forms of the disease characterized by a relatively low or absence of inflammatory cells. In addition, most of the drugs have side effects of their immunosuppression. Therefore, treatment strategies aimed at preventing neurodegeneration are essential to develop, in order to attenuate progression of disability and perhaps to prevent the transformation of the clinical phenotype relapsing remitting to secondary progressive disease. Research on biomarkers for evaluation of the bioavailability of drugs use are needed

Neuroprotection

It has become clear that the pathophysiology of MS is not adequately explained by acute focal inflammatory attacks inside the CNS. We need to take into account the

neurodegenerative process of axonal loss, which may partly occur independently from inflammation and certainly arise already early in the disease process. If inflammation and neurodegeneration act as two independent processes, strategies to protect the neuron should be developed in MS. However, neurodegeneration in MS is probably a complex process and the involvement of demyelination and inflammation by for example CD8 positive cells to axons and secretion of toxic factors will be involved. Other factors, which may be involved, are excitotoxicity by glutamic acid binding to excitatory amino acid receptors on cell antibodies, dendrites and axon terminals of neurons initiating necrotic cell death (Ziemssen et al., 2005).

Therefore, treatment in MS should focus on suppression of the inflammatory process and restoration and protection of glial and neuronal function.

The potential neuroprotective function of inflammation is relevant in the treatment goals. This neuroprotectivity may be explained by the release of neurotrophic factors from immunecells that promote neuronal repair or protect against injury. Neurotrophic factors involved are brain-derived neurotrophic factor (BNDF) in humans and in experimental allergic encephalitis (the mouse model of MS) nerve growth factor, leukaemia inhibitory factor and glial growth factor 2 delayed the onset of disease or reduced the severity of the neurological deficit (Ziemssen et al., 2005). Another interesting treatment strategy is the delivery of neuroprotective factors by retroviral transduction of these neurotrophic factors into antigen-specific cell lines (Kramer et al., 1995). Axonal protection has been demonstrated experimentally using glutamate antagonists (Pitt et al., 2000; Smith et al., 2000), sodium channel blockers (Bechtold et al., 2004; Kapoor et al., 2003; Lo et al., 2003), Calcium channel blockers (Brand-Schieber et al., 2004), blockers of sodium calcium exchange (Kapoor et al., 2003), cannabinoid receptor agonists working partly through the glutamate system (Pryce et al., 2003) and free radical scavengers (Hendriks et al., 2004). Clinical trials will have to test these potent treatment options.

Immunosuppressive therapy seems to become critical when the harmful effects of the inflammatory reaction outweigh the beneficial effects. To detect that point in the disease when the immunomodulatory treatment will fail, because the inflammation is more beneficial than harmful is difficult to establish. Probably combined approaches of neuroprotection and immunosuppresion will be beneficial.

Limitations of biomarker research in MS

Despite numerous of studies on biomarkers in MS, the number of markers that have emerged as clinically useful is unfortunately still rather small. Often, initially reported studies of a marker show great promise, but subsequent studies on the same or related markers yield inconsistent conclusions or stand in direct contradiction to the promising results. It is imperative that we attempt to understand the reasons that multiple studies of the same marker lead to differing conclusions. A variety of problems have been cited to explain these discrepancies, such as general methodologic differences, poor study design, assays that are not standardized or lack reproducibility, and inappropriate or misleading statistical analyses that are often based on sample sizes too small to draw meaningful conclusions. For example, especially in retrospective studies, patient populations are often biased toward patients with available specimens. Some parts of this thesis we have used CSF, which might best reflect the pathology ongoing in the central nervous system, but unfortunately its availability is low, since a lumbar puncture is an invasive procedure and only limited amounts of CSF can be drawn. The patients selected for our studies were biased in the way that they voluntarily underwent a lumbar puncture. Another way to collect CSF is to ask people who are undergoing a spinal tab for clinical purposes to participate in the study, but this will then bias studies to include patients who are in the beginning of the disease. Off course, blood is a more accessible body fluid, but systemic fluctuations are more likely to influence the result. Tears have been mentioned to reflect the CSF, but are only available in small amounts. Urine samples are often “dirty” and therefore often not suitable to develop a clinical relevant biomarker assay on.

The selection of patients should ideally represent a group or subgroup of the patients seen in daily clinical practice. Often, due to the minimal availability of specimen and bad categorisation of subgroups, useful information is lost. Subjective interpretation of clinical progression is used, instead of relying on more objective clinical scales and MRI measurements. The reporting of therapy used in the patients selected should be available to interpret the data in an accurate manner.

Specimen availability may be related to patient outcome, and the quantity, quality, and preservation method of the specimen may affect feasibility of conducting certain assays. There can also be biases or large variability inherent in the assay results,

depending on the particular assay methods used. For example Western blots are very subjective to inter-pretation.

Statistical problems are commonplace. These problems include underpowered studies or overly optimistic reporting of effect sizes and significance levels due to multiple testing, subset analyses, and cut point optimization. This is not only the case in MS biomarker research, but has been shown also in other fields of medical research on markers (McShane et al., 2005).

Future perspectives

Future research should focus on identification of biomarkers that reflect the ongoing pathology or pharmacological responses to an intervention in individual patients. The *in-vivo* pathological processes ongoing in MS lesions can only be obtained by examination of tissue. This is hard to obtain from living patients. The most accepted method these days to classify the pathology ongoing in brain and spinal cord is the combined assessment of clinical (subtype, disability) and MRI features (the appearance of the lesions, gadolinium enhancement, black holes, atrophy, reduction on N-acetylaspartic lesions). As mentioned before when discussing the limitations of biomarker research, the development of clinical scales, to optimize the identification of MS subtypes, and of new MRI measurements to better reflect the pathological features is needed. Furthermore, additional markers in body fluids are needed to further identify the heterogeneity in individual MS patients. Therefore we should measure and evaluate biomarkers and classify them in relation to the process they are reflecting: immune system, Blood-brain-barrier disruption, demyelination, oxidative stress and excitotoxicity, axonal and neuronal damage, gliosis and remyelination and repair (Bielekova et al., 2004). Furthermore we should try to establish the exact mechanisms and relationship between these processes.

Till now biomarker research has been mainly focused on immune system markers. This has revealed several interesting candidates such as cytokines and chemokines (as well as their receptors). Both of them participate in controlling trafficking of cells and cause inflammation in the CNS. Further studies to reveal the exact mechanisms, their possible roles in classification, disease activity and prediction of clinical course in MS

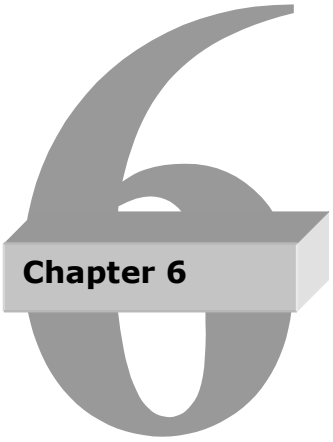
are needed. In order to achieve this we should look for immunological *patterns* (cytokines and chemokines) in different subgroups and relate them to MRI features. There seems a dual nature of inflammation, both detrimental and protective, so it would not be realistic to focus on a single immunological marker.

The biomarker search for neurodegeneration has only just started and has already given some promising results. Neurofilaments (Petzold et al., 2005, Petzold et al. 2004; Malmestrom et al., 2003), GFAP (Norgren et al., 2004; Malmestrom et al., 2003) and GAP43 (Teunissen et al., 2006) have given interesting data. Further evaluation of how well these markers reflect axonal damage as visualised on clinical and MRI characteristics, or relate to disease progression rate has to be further evaluated in larger studies.

Furthermore, we should screen with novel techniques at different levels of expression (genomic, transcriptomic, proteomic and metabolic level). Proteomics is a promising field in the biomarker research. Proteomic technologies such as immunoblotting, isoelectric focusing, 2D gel electrophoresis and mass spectrometry have proven useful for identifying a unique proteome. An advantage of this field is that only very small aliquots of body fluid are needed. However, proteomics will generate a lot of data, which can be hard to interpretate. It will give us not only data on the CNS, but also from peptides, proteolytic fragments and antibodies that can cross the blood–brain barrier. While often the Blood-brain-barrier in MS is disturbed this will influence the interpretation of the data and the relevance of a given protein. In addition, caution is needed while the proteins identified by immunoblotting are most of the time high abundance proteins and the identification of the proteins may be incorrect by incomplete databases of the mass spectrometry. Probably proteomics should be best seen as a robust tool to identify potential candidate proteins for further study.

To generate enough power for biomarker studies, collaboration between different multiple centres is of uppermost importance. This underlines the need for standardisation in methods used for sample collection, clinical scores and MRI scan protocols. Long-term follow up of serially collected clinical, radiological, and neurodegenerative and immunological parameters will facilitate longitudinal studies. Sharing and comparing data and validating new candidate biomarkers would improve the comparability between the centres. Well-defined controls should be collected to give reliable reference values. Hopefully, further efficient use of the available and upcoming research will eventually lead to optimal biomarkers, which can be used in

clinical practice to discriminate subgroups, guide doctors in current treatment strategies and thereby preventing axonal injury and thus so irreversible disability in MS patients.



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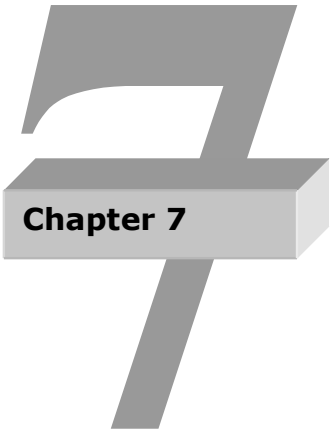

Samenvatting:

Biologische markers in multipele sclerose, gerelateerd aan ziekteactiviteit en progressie

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Multipele sclerose (MS) is een chronische inflammatoire aandoening van het centraal zenuwstelsel (CZS). De ziekte veroorzaakt demyelinisatie en verlies van axonen in hersenen en ruggenmerg. Dit kan leiden tot beschadiging van de zenuwgeleiding en achteruitgang van de neurologische functies. Ondanks uitgebreid onderzoek is tot op heden de oorzaak van de ziekte nog niet bekend.

In **hoofdstuk 1 (Inleiding)** wordt nader ingegaan op de achtergronden van MS. Verschillende histopathologische patronen en klinische beloopsvormen zijn beschreven, die suggereren dat MS een heterogene ziekte is. Genetische en omgevingsfactoren lijken daarbij de gevoeligheid voor de ziekte te bepalen. De inflammatie in MS is het gevolg van een immunologische reactie, waarbij autoreactieve T cellen een belangrijke rol spelen in de initiatiefase van het proces. Deze T cellen komen vanuit het perifere bloed en passeren de bloed-hersen-barriere, waarna er na het herkennen van het antigeen een immuno-logische reactie volgt. Hierbij zijn diverse componenten van het immuunsysteem betrokken, met onder andere vrijkomen van cytokines en chemokines. De inflammatoire reactie is geassocieerd met vorming van demyeliniserende plaques, waarbij de axonen relatief gespaard worden. Naast deze plaques is er echter ook macroscopisch normaal uitziende witte stof, waarbij bij histopathologisch onderzoek sprake blijkt van diffuse schade, gekarakteriseerd door axonaal verlies en activatie van microglia en astroglia. Dit suggereert dat naast de inflammatie, een neurodegeneratief proces een rol speelt. De relatie tussen deze beide processen (inflammatie en neurodegeneratie) enerzijds en de verschillende ziektestadia anderzijds, is onbekend. Het verkrijgen van inzicht in deze processen in verschillende MS subgroepen, zal hopelijk uiteindelijk leiden tot verbetering van de behandeling. Het doel van dit proefschrift is om beter inzicht te krijgen in de verschillende pathologische processen betrokken bij MS. Daartoe is de relatie onderzocht van zowel immunologische biomarkers als biomarkers die de

schade aan het centraal zenuwstelsel reflecteren enerzijds, met de klinische en magnetische resonantie imaging (MRI) parameters anderzijds.

In **hoofdstuk 2** worden de studies beschreven die de relatie onderzoeken tussen immuun metingen en klinische ziektebeloop, evenals de lange termijn ziekte progressie op MRI brein-scans.

Hoofdstuk 2.1 worden de resultaten van een longitudinaal onderzoek gerapporteerd, die de expressie van chemokinereceptoren (CCR5 en CXCR3) op Tcellen laten zien in 124 MS patienten behorend tot verschillende subgroepen. Hogere percentages CD8 positieve cellen worden gevonden bij de secundair progressieve (SP) vorm, in vergelijking met de primair progressieve (PP) vorm van MS. PP patienten blijken een hogere expressie CCR5 CD 8 positieve cellen te hebben in vergelijking met relapsing remitting (RR) patiënten. De resultaten van het onderzoek suggereren een hogere CD8 celexpressie van receptor CCR5 in progressieve (SP en PP) MS patiënten in vergelijking met RR patiënten. De voor-spellende waarde van (1) CXCR3 expressie op CD8 positieve cellen in de gehele groep, (2) de CXCR3 expressie op CD4 en CD8 positieve cellen (in SP) en (3) de CCR5 expressie op CD8 positieve cellen (in RR), voor de jaarlijkse veranderingen in T2 laesies worden in dit onderzoek bevestigd. Deze resultaten suggereren dat de twee ligand/ receptorparen CCL5/CCR5 en CXCL10/CXCR3 een rol spelen bij de actieve vorm van de ziekte, omdat de expressie van CCR5 en CXCR3 op T lymphocyten de toekomstige activiteit van de ziekte voorspellen zoals gemeten op MRI-scans. Aangezien de correlaties van de chemokinereceptor expressie met name significant zijn met veranderingen in T2 laesies (nieuwe laesies voorstellende) en niet of minder met T1 laesies (voorstellende meer destructieve laesies), lijkt de chemokine-as relevanter voor de initiatie van de laesies dan voor de uitkomst van de laesies op de lange termijn.

In **hoofdstuk 2.2** wordt een cross-sectionele studie naar de chemokines CXCL10 en CCL2 in bloed en cerebrospinaal vloeistof (CSF) beschreven in 51 MS patiënten. Deze markers zijn gerelateerd aan klinische en MRI metingen van de ziekteactiviteit. Hierbij worden geen verschillen gevonden tussen de verschillende subgroepen en evenmin worden er significante correlaties gevonden tussen de chemokines en de klinische en MRI data. Dit houdt in dat in klinisch stabiele MS patiënten, de chemokines CXCL10 en CCL2 gemeten in serum en CSF geen bruikbare markers zijn voor de ziekteactiviteit in MS.

In **hoofstuk 2.3** wordt een longitudinale studie beschreven waarbij is onderzocht of de expressie van een aantal adhesiemoleculen (VLA-4, LFA-1, ICAM-1) op T lymphocyten kan onderscheiden tussen de subgroepen van MS, als ook de voorspellende waarde er van voor de ziekteactiviteit, zoals gemeten op MRI scans van de 124 eerder genoemde MS patienten. Significante verschillen zijn gevonden tussen de klinische subgroepen voor de CD11a en CD18 expressie op T cellen, evenals voor de CD49d+ CD8+cellen en CD29+ T cellen. De ziekteprogressie op MRI (Δ T2 laesies) is significant gecorreleerd met de volgende adhesie moleculen: CD29 op CD4+ cellen; CD18 op CD4+ cellen en CD8+ cellen; CD49 op CD4+ cellen. Deze correlaties zijn sterker in de SP populatie. In RR MS patiënten correleren de T1 laesies significant met CD49d op CD4+ cellen. Deze resultaten laten zien dat met name de integrines een belangrijke rol spelen in de inductiefase van de ontwikkeling van laesies in MS.
In **hoofdstuk 2.4** worden in een cross-sectionele studie bij MS (n=124) en controle (n=34) patiënten sexe verschillen beschreven in expressie van cytokines in CD4+ en CD8+ T cellen. Hoewel er geen verschillen worden gevonden tussen mannen en vrouwen in de gehele MS groep, blijken vrouwen in de progressieve fase van de ziekte hogere en in de relapsing remitting fase lagere hoeveelheden pro-inflammatoire cytokines te hebben in vergelijking met mannen. Dit impliceert dat cytokine producerende T cellen en sexe verschillen in cytokine productie kunnen worden gevonden in de diverse stadia van de ziekte, die mogelijk gerelateerd zijn aan het onderliggende ziektemechanisme.
In **hoofdstuk 2.5** is onderzocht of opticospinale MS (OSMS) verschilt van klassieke MS (CMS) met betrekking tot immuun markers die tot expressie komen op perifere T cellen. In vergelijking met CMS, wordt bij OSMS een lager percentage CD4+ T cellen welke Th1 cytokine IFN γ produceren en hogere percentages CD8+ T cellen, welke Th2 cytokine IL10 produceren geobserveerd. Deze bevindingen geven aan dat er bij OSMS een shift is in de richting van een Th2 respons. Dit is in lijn met de hypothese bij de ziekte van Devic's (neuromyelitis optica), een ziekte lijkend op opticospinale MS.

In **hoofdstuk 3** van dit proefschrift worden biologische markers betrokken bij degeneratie van het centraal zenuwstelsel onderzocht bij verschillende klinische en paraklinische subgroepen.

Hoofdtuk 3.1 rapporteert onderzoek naar de relatie tussen de concentratie van biomarkers voor de glia reactie in de CSF van 51 MS patiënten van verschillende klinische subtypes en de mate van invaliditeit, waarbij de bevindingen met een post-mortem studie werden gevalideerd. Een trend voor toenemende S100B concentraties van PP naar SP naar RR wordt waargenomen, terwijl de ferritine concentraties hoger zijn in SP dan in controle patiënten. De S100B: ferritine ratio discrimineert patiënten met RR MS van SP, PP of controles. MS patiënten met veel invaliditeit hebben in hun CSF hogere GFAP concentraties, in vergelijking met mindere geinvalideerde MS of controle patiënten. De post-mortem studie laat hogere concentraties S100B in de acute in vergelijking met de subacute plaques zien, terwijl de ferritine concentraties verhoogd zijn in alle MS laesies, ongeacht hun stadium. Concluderend, lijkt S100B geassocieerd te zijn met de relapsing fase van de ziekte, terwijl ferritine gedurende het gehele ziektebeloop relatief verhoogd is. GFAP is positief gecorreleerd met de invaliditeits-schalen en kan een goede maat zijn voor de irreversibele schade.
In **hoofdstuk 3.2** worden de neurofilamenten lichte (NfL) en zware keten (NfH) en hun respectivelijke antilichamen in gepaarde CSF en serum samples van patienten met verschillende subgroepen van MS, geëvalueerd in relatie tot verschillende MRI parameters voor weefselschade. Neurofilament zware en lichte keten en hun antilichamen kunnen daarbij geen onderscheid maken tussen de subgroepen. De anti-NfL index (CSF anti-NfL units/serum units)/ (CSF albumin/serum albumin*)* correleren significant met al.le markers voor hersenschade (parenchymale (PF) and ventriculaire fracties, T1 en T2 laesies). Voor de anti-NfH index is er een significante relatie met de atrofie marker PF. Deze studie kan dan ook richting geven aan verder onderzoek naar biomarkers, die gerelateerd zijn aan de *in vivo* MRI pathologie.
In **hoofdstuk 3.3** is een longitudinale studie verricht bij 28 MS patienten en 9 controle personen en hun CSF concentraties van de gephosphoryleerde (NfH p) en de uitgebreid gephosphoryleerde neurofilament zware keten (NfH ep) bepaald. Concentraties van NfH phosphovormen, de mate van phosphorylatie (ratio van NfH phospho-vormen) en veranderingen in NfH concentraties tussen baseline en follow-up zijn gerelateerd aan de klinische phenotypen (groepen) en invaliditeitsschalen. Driekwart van de patiënten met een progressief beloop hebben een verhoging van NfH p concentratie tussen baseline en follow-up, terwijl dit eveneens voorkomt bij éénvijfde van de RR groep. Progressieve patienten hebben hogere concentraties van NfH p ten opzichte van stabiele RR MS patienten op baseline en follow up tijdstippen.

Mediane NfH p concentraties op baseline waren hoger bij klinisch progressieve RR versus klinisch stabiele RR MS patienten.
NfH p correleerde met de klinische schalen bij follow up. Deze studie laat zien dat CSF Nf phosphovormen voorspellers zijn voor de neurologische schade in RR MS en suggereert dat vroege axonale schade een slecht prognostisch teken is. Gedurende de progressieve fase van de ziekte wordt een verhoging van Nf concentratie geobserveerd, hetgeen een cumulatieve axonale schade suggereert.

Conclusies die kunnen worden getrokken uit dit proefschrift zijn:

- Immunologische markers op periphere T cellen, met name chemokine receptoren (CXCR3 en CCR5) en adhesie moleculen (met name integrines), hebben een voorspellende waarde voor het ontwikkelen van laesies;
- Verschillen tussen de sexen in cytokine productie van T cellen zijn aanwezig in de verschillende ziektestadia;
- Opticospinale MS lijkt een Th2 gemedieerde ziekte, zoals ook wordt verondersteld bij neuromyelitis optica;
- Het bepalen van de glia activiteit, zoals gereflecteerd door S100B en GFAP in CSF, kan helpen te differentiëren tussen de subgroepen in MS;
- Neurofilament antilichamen lijken *in vivo* pathologie op de MRI te weerspiegelen;
- CSF Nf phosphoforms zijn voorspellers voor de neurologische schade en invaliditeit, met name in de relapsing remitting fase van de ziekte.

Tot slot wordt in **hoofdtuk 5** een overzicht gegeven van de huidige kennis met betrekking tot inflammatoire en neurodegeneratieve biomarkers, de klinische relavantie en suggesties voor toekomstig onderzoek naar biomarkers in MS.

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Curriculum Vitae

Judith Eikelenboom werd op 7 januari 1971 geboren in Woudenberg. Zij bezocht het Revius Lyceum te Doorn en deed in 1989 eindexamen. Hierna studeerde zij aansluitend een jaar Frans aan de Universiteit van Nantes te Frankrijk. Vervolgens startte ze met haar studie Geneeskunde aan de Universiteit van Amsterdam, waar ze in 1995 haar doctoraal behaalde. De onderzoekstage aan het eind van haar studie deed ze op de afdeling klinische en experimentele cardiologie in het Academisch Medisch Centrum te Amsterdam (hoofd: Prof. Dr. M.J. Janse). Tijdens haar co-schappen deed ze onderzoek naar de effecten van glucocorticoiden op de hypothalumus bij het Nederlands Instituut voor Hersenonderzoek te Amsterdam (hoofd: Prof. Dr. D.F. Swaab). In 1998 behaalde ze het artsexamen en werkte ze vervolgens ruim een jaar als arts-assistent op de afdeling neurologie van het Medisch Centrum Alkmaar (hoofd: Dr. R. ten Houten).

Onder leiding van Prof. Dr. C.H. Polman en Dr. B.M.J. Uitdehaag begon ze in 2000 met haar promotieonderzoek naar biomarkers in de ziekte Multipele Sclerose op de afdeling neurologie van het VU Medisch Centrum in Amsterdam. Tijdens deze periode heeft zij tevens onderzoek gedaan op de afdeling neurologie van het University of Colorado Health Sciences Center te Denver in Amerika (hoofd: Prof. Dr. D.H. Gilden). In 2003 startte ze de opleiding tot neuroloog in het VU Medisch Centrum (hoofd: Prof. Dr. J.J. Heimans).

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List of abbreviations

AI	ambulation index
AL	acute lesions
APC	antigen presenting cells
APP	amyloid precursor protein
BBB	blood brain barier
BSP	brain-specific proteins
CL	chronic lesions
CNS	central nervous system
CR	chemokinereceptor
CSF	cerebrospinal fluid
CV	coefficient of variation
DMT	disease modifying treatment
EAE	experimental autoimmune encephalomyelitis
EDSS	expanded disability status scale
GFAP	glial-fibrillary acidic protein
GM	grey matter
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IQR	interquartile range
LFA	lymphocyte function associated antigen
LL	lesion load
LP	lumbar puncture
LPS	lipopolysaccharide
MAB	monoclonal antibody
MRC	medical research council
MRI	magnetic resonance imaging
MS	multiple sclerosis
MHC	major histocompatibility complex
MMP	matrix metalloproteinases
MSFC	multiples sclerosis functional composite
NAWM	normal appearing white matter
Nf	neurofilaments
NfH	neurofilaments heavy chain
NfL	neurofilaments light chain
NfM	neurofilaments medium chain
NMO	neuromyelitis optica
OSMS	opticospinal multiple sclerosis
PBMC	peripheral blood mononuclear cells
PF	parenchymal fraction
PP	primary progressive
RR	relapsing remitting
SAL	subacute lesions
SD	standard deviation
SEM	standard error of the mean
SP	secondary progressive
TCR	T cell receptor
Th	T helper
TNF	tumour necrosis factor
VCAM	vascular adhesion molecule
VEP	visual evoked potential
VF	ventricular fraction
VLA	very late antigen
9HPT	9-hole PEG test